LIVER INJURY

Transactions of the Twelfth Conference
September 21, 22 and 23, 1953, Princeton, N. J.

Edited by
F. W. HOFFBAUER, M.D.
ASSOCIATE PROFESSOR, DEPARTMENT OF MEDICINE
UNIVERSITY OF MINNESOTA HOSPITALS
MINNEAPOLIS, MINN

Sponsored by the
JOSIAH MACY, JR FOUNDATION
NEW YORK, N Y

Library of Congress Catalog Card Number. 48-4371
Price $4 25

Printed in the United States of America
By Corlies, Macy & Company, Inc, New York, N Y

PARTICIPANTS

*Twelfth Conference on Liver Injury**

MEMBERS

CHARLES H BEST, *Chairman*
Banting and Best Department of Medical Research, University of Toronto
Toronto, Canada

F W. HOFFBAUER, *Secretary*
Department of Medicine, University of Minnesota Hospitals
Minneapolis, Minn

JESSE L BOLLMAN
Department of Physiology, The Mayo Foundation Graduate School, University of Minnesota
Rochester, Minn

HARRY GOLDBLATT
Mt Sinai Hospital
Cleveland, Ohio

PAUL GYÖRGY
Department of Clinical Pediatrics University of Pennsylvania School of Medicine
Philadelphia, Pa

FRANKLIN M HANGER
Department of Medicine, Columbia University, College of Physicians and Surgeons
New York, N Y

W STANLEY HARTROFT
Banting and Best Department of Medical Research, University of Toronto
Toronto Canada

MELVIN H KNISELY†
Department of Anatomy The Medical College of the State of South Carolina
Charleston, S C

SIDNEY C MADDEN
Department of Pathology, University of California School of Medicine
Los Angeles, Calif

JOHN R NEEFE
Department of Medicine, University of Pennsylvania Hospital
Philadelphia, Pa

ARTHUR J PATEK, JR †
Goldwater Memorial Hospital, Welfare Island
New York, N Y

HANS POPPER
Hektoen Institute for Medical Research, Cook County Hospital
Chicago, Ill

EPHRAIM SHORR
Department of Medicine, Cornell University Medical College
New York, N Y

---

* This is the final conference
† Absent



1st Row, Cecil J. Watson, Franklin M. Hanger, [illegible]

TABLE OF CONTENTS

Twelfth Conference on Liver Injury

THE JOSIAH MACY, JR. FOUNDATION
CONFERENCE PROGRAM

tions" more evident than in medicine. Today, to be effective, medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other, for advances in one field are frequently dependent upon knowledge derived from quite another discipline.

Although the fertility of the multidiscipline approach is thus recognized, universities, and scientific societies and journals which are usually restricted to one small area of a field in their coverage, have not yet made adequate provision for channels of interdisciplinary communication. We do not wish to compete with the formal scientific meetings or with the scientific journals which have established patterns and formats for the presentation of material Our purpose at the meetings is to keep an informal atmosphere and to encourage the exchange of methods, research plans, concepts and difficulties, which cannot be done if there is formal speech making.

The Foundation has endeavored to meet the need for interdisciplinary communication by bringing together for a series of two-and-a-half day annual conferences a small group of investigators, representing in so far as possible all the branches of science related to a chosen problem Participants in these informal conferences over a five-year period develop a feeling of friendship, trust and mutual respect which in turn promotes communication, cross-fertilization of ideas and cooperation The success of such an endeavor, however, is dependent upon full participation of all members in the discussion Accordingly attendance at any conference is limited to twenty-five

Under the guidance of Dr Willard C. Rappleye, President of the Foundation since 1942, the Conference Program has been gradually expanded and enlarged until during 1953 it included twelve different groups which meet annually to discuss a wide variety of problems in the field of medicine and the closely related disciplines Our plan is to discontinue the meetings of each group at the end of five years.

In order to share with a wider group of investigators and students the essential quality of these conferences and to give others an insight into the functions of the scientific mind, the informal nature and tempo of the discussions, as far as possible, are preserved in the published transactions

Frank-Fremont Smith, M D,
Medical Director

THE LIVER AND CARBOHYDRATE METABOLISM

EINAR LUNDSGAARD
Department of Physiology
Institute of Medical Physiology
University of Copenhagen
Copenhagen, Denmark

I HAVE BEEN asked to make some introductory remarks on liver and carbohydrate metabolism. They are closely related: the liver plays a role in carbohydrate metabolism, and at the same time carbohydrate metabolism has a specific significance for the metabolism of the liver.

The liver functions as a store or depot of carbohydrate and is the main, if not the only, site of glyconeogenesis. These are the two principal factors in the part played by the liver in carbohydrate metabolism. Other points of minor importance might, of course, be mentioned. When I stated that carbohydrate metabolism was of significance for the metabolism of the liver, I was, of course, hinting at the antiketogenic effect of carbohydrates.

I shall not, at this juncture, attempt to give a review of the very comprehensive subject on which I am speaking, but shall confine myself to a presentation based on the experiences gained in my laboratory. Therefore, it will necessarily be rather one-sided, particularly because most of our experiments have been performed on artificially perfused and isolated cat livers. However, perhaps just for that reason it will stimulate discussion.

I shall not say much about techniques, but I think I ought to mention that we always used blood from the same species in our perfusion experiments. As a rule we used defibrinated blood slightly diluted with Ringer solution, but now and then we used heparinized blood. I do not think it makes any difference. The blood was oxygenated with oxygen containing about 4.5 per cent carbon dioxide, the liver was perfused only from the portal vein. We have occasionally perfused simultaneously from the hepatic artery without noticing any difference in the behavior of the liver.

The perfusion technique has certain advantages which scarcely need to be pointed out, but it also has drawbacks. It can be claimed

that the artificially perfused liver is not in a normal state. I could present arguments in favor of this claim, but I shall confine myself to mentioning that the isolated liver always and steadily gives off inorganic phosphate to the blood. This undoubtedly must signify some kind of impairment. I mention this regular observation also because it has some bearing on the carbohydrate metabolism of the isolated liver.

Fremont-Smith: Dr. Lundsgaard, will you tell us about the pressure relationships in your perfusion? How much pressure did you use, and how much liver?

Lundsgaard: A cat liver generally weighs from 70 to 80 gm. The flow is usually from about 60 to 65 ml. per minute, which gives a reasonable arteriovenous difference in oxygen content of the blood. In a good preparation, this flow can be upheld with a pressure of from 10 to 12 cm H_2O.

Fremont-Smith: Is it steady flow, or is it pulsating?

Lundsgaard: It is pulsating.

Hanger: May I ask if the liver you perfuse gives off potassium as well as phosphate?

Lundsgaard: I have never dared make potassium determinations because it is practically unavoidable to have a slight hemolysis in the blood in a perfusion experiment. Even though it is slight it occurs and will influence the potassium concentration in the plasma.

Shorr. Did that affect the phosphate values you obtained?

Lundsgaard. I do not think so, because as I shall mention later, under certain circumstances the steady increase in plasma phosphate can be inverted

It is an old observation in our experiments that there is a striking difference between the rabbit and cat livers in their ability to store glucose as glycogen. When a rabbit liver is perfused with blood with fairly high glucose concentration, the glucose concentration in the blood diminishes rapidly and glycogen is stored in the liver. Even at high blood sugar concentrations, a fall in blood sugar concentration is never observed in experiments on cat livers. In such experiments, a slight but steady increase in the blood glucose concentration is always observed As this holds true even when working with practically glycogen-free livers, the increase in blood sugar concentration must undoubtedly signify a glyconeogenesis

In spite of the striking difference in carbohydrate metabolism, as roughly judged from the changes in blood sugar concentration, the just mentioned steady loss of phosphate is met with in the liver from both species If phosphate acceptors with apparently high

avidity, such as fructose or glycerol, are added to the blood perfused through a cat liver, the concentration of inorganic phosphate in the plasma falls steeply. The effect on the plasma phosphate is only transitory. As soon as the added phosphate acceptor has disappeared from the blood, the phosphate concentration rises steeply and reaches the same concentration as would have been reached without any addition to the blood Whether the same effect on the blood phosphate concentration can be evoked in the rabbit liver by adding fructose or glycerol, I do not know. It ought perhaps to be looked into. At any rate, I think it can be stated that glucose is not a sufficiently greedy phosphate acceptor in the rabbit liver to prevent the constant loss of phosphate The loss in the cat liver consequently cannot be attributed to its apparent inability to store glucose as glycogen

The amount of phosphate given off from such a liver is rather small. We generally use a volume of, say, 250 ml of blood with about 150 ml of plasma During an experimental period of about 1 5 hours, the concentration of inorganic phosphate in the plasma increases from 4 to 5 mg per cent at the start of the period to 9 or 10 mg per cent at the end That is a rather marked increase in concentration, but the amount of phosphate given off from the liver is small

As already hinted, fructose is metabolized in the isolated cat liver with great avidity Half an hour after the addition of from 1 to 1 5 gm of fructose to the blood, the fructose has disappeared. Generally half of the amount added has been stored as glycogen, and the other half reappears in the blood as glucose. I shall, however, not go further into this peculiar feature in the carbohydrate metabolism of the cat liver

I think Dr Kosterlitz stated that the diabetic liver stores fructose as glycogen, but does not touch glucose We may say that the isolated cat liver behaves as a diabetic liver, but with the difference that it cannot be influenced by insulin

Shorr. How high do the glucose levels rise? I had in mind the work of Major and Mann (1) and also Barker and Sweet (2) on depancreatized dogs These workers demonstrated that when high levels of glucose were maintained in the blood stream of diabetic dogs, there was a significant storage of glycogen in the liver, and also in the muscles Barker and Sweet showed that this took place without any oxidation of carbohydrate in the diabetic animal Is my recollection correct, Dr Best?

Best Yes, in diabetic animals.

Shorr: And in phlorhizined animals

Lundsgaard: I have never worked with quite so high a concentration, but I have performed experiments with isolated cat livers over a wide range of glucose concentrations. In some experiments, I have even artificially lowered the glucose concentration for special purposes. The highest concentration has been from 500 to 600 mg. per cent. Do you remember how much glycogen was deposited in Soskin's experiments with glucose?

Shorr: I cannot cite figures, but they were very significant.

Kosterlitz: As far as I remember, I gave equal quantities of glucose and fructose to diabetic dogs, and obtained from 0.2 to 0.3 per cent liver glycogen with glucose, and up to 6 per cent with fructose. The difference was as considerable as that (3).

Shorr: My recollection is that it was distinctly above your lower figures, but not as high as your higher ones.

Best: I think quite a bit of doubt has been thrown on some of those experiments, because no one knows how long a little insulin persists after removal of the pancreas. Recent work from Cori's laboratory (4) suggests that even three or four days after complete removal, we can find some indication that a few active molecules of insulin are still there.

Shorr: Of course, in the case of phlorhizin, there is the question of how long glucose can be perfused without bringing about the eventual stimulation of the pancreas that comes from exposures to glucose. Therefore, I think both experiments may be vulnerable.

Kosterlitz: In my experiments, fructose was given weeks and months after pancreatectomy

Best. I think it is a great admission of ignorance that we have not been able to perfuse an isolated organ and have it perform its physiological functions Therefore, Dr Lundsgaard's statements about physiological results in the isolated liver are extremely interesting.

Lundsgaard Nevertheless, I should like to point out that I do not consider this as an absolutely normal state Under ordinary conditions, and even at high blood glucose concentrations, no net carbohydrate metabolism is demonstrable in the cat liver by determinations of liver glycogen and blood sugar concentration. I may, in this connection, mention that the respiratory quotient of the isolated cat liver is always very low, generally between 0.6 and 0.7.

We have, of course, endeavored to induce a net carbohydrate metabolism, especially a deposition of glucose as glycogen, in the cat liver I may at once say that we have utterly failed I shall not

go into the different measures which we have tried I shall only dwell on our failure to evoke a deposition of glycogen in the isolated cat liver by adding insulin in various doses to the blood Nor has it been possible in experiments on rabbit livers to demonstrate an

f glycogen
igh blood
f glycogen
en added

On the other hand, at corresponding blood sugar concentrations, insulin has a very marked effect on the utilization of glucose in a hind limb preparation Our failure to demonstrate an effect of insulin on isolated livers has of course raised the question as to whether insulin exerts any direct effect on the intact liver It cannot be doubted that it is possible to induce storage of glycogen in the glycogen-poor liver of a diabetic organism by administration of insulin, but this may be a secondary effect, the mechanism of which we cannot account for.

The question of whether insulin promotes glycogen deposition in the liver of the normal organism has been answered in contradictory ways by different investigators Offhand, one would think it easy to obtain an answer to this question by simple experiments However, there is the difficulty that the hypoglycemia caused by insulin must be assumed to evoke a liberation of epinephrine, which again counteracts a possible effect of insulin on the glycogen content of the liver. When a decline in glycogen content in the liver is observed after administration of insulin, it is generally interpreted in this way

Shorr How much time elapses from the removal of the cat liver to the termination of the experiment?

Lundsgaard. I usually allow from 20 to 30 minutes for the isolated liver to settle down to the changed condition A half hour after the actual isolation, we start our experimental period We then generally carry on an experiment from 1½ to 2 hours. The liver will undoubtedly keep in good shape for somewhat longer, but I prefer not to extend the actual experimental period beyond about 2 hours. That is about 2½ hours for the artificial perfusion

Shorr The reason I raised the question was to bring up the possibility that we may be dealing with an escape from anti-insulin factors in the tissue which comes about with time. The function of insulin, broadly speaking, is to counteract the effect of anti-insulin factors on carbohydrate metabolism, which can go on independent of insulin in tissue provided these anti-insulin factors are not operating The deterioration of these factors in tissue takes place with time

on incubation of tissue *in vitro;* hence it is important to consider this process in any prolonged *in vitro* experiments. However, I think that the time periods over which your experiments were carried out were too short to introduce this factor to a significant degree.

Fremont-Smith: You are referring to your experiments on the heart?

Shorr: I am referring to some experiments I carried out in 1937 (5) with cardiac slices removed from dogs with pancreatic diabetes. These slices have an initial *in vitro* respiratory quotient (R.Q.) of from 0.70 to 0.73 If they are then incubated in Ringer glucose phosphate in an atmosphere of oxygen at 37 5° C. with shaking, their R.Q will rise to 0.85 at the end of 5 hours and to 1 00 at the end of 10 hours. If we work at 41° C, the diabetic heart slices have a R.Q of 1.00 at the end of 4 hours If we add insulin to the diabetic slices, we do not accelerate the rate at which these slices have an elevation of R.Q. I interpreted these phenomena as indicating that in the diabetic heart in which insulin was absent, there were anti-insulin factors present presumably of pituitary and/or adrenal origin. With the prolongation of incubation these factors were destroyed and the diabetic heart was now free of the anticarbohydrate action and could resume their inherent potentialities for oxidizing glucose without the intervention of insulin

Lundsgaard Insulin is usually added in the middle of the actual experimental period, that would be within 1½ hours after the liver has been isolated.

Best In general, in hypophysectomized animals, where the thyroid, adrenals, and gonads have degenerated – a little activity may be left, but not much – we can still demonstrate all the actions of insulin beautifully. the storage of glycogen and fat, and the retention of nitrogen However, it is not a perfect physiological experiment, and I think we probably should attempt to get rid of all those antagonistic factors. My prediction would be that the action of insulin would be obtained just as dramatically even though the experiment was not sound from a physiological point of view.

Lundsgaard I think the last point I mentioned was this question of the effect of insulin on the glycogen content in normal, not diabetic, animals

Best We are all probably thinking that many of these experiments were done before the hypoglycemic factor was separated from insulin.

Lundsgaard Even in our older experiments, we used crystalline insulin which we obtained from Dr John J. Abel in Baltimore At

the same time, we had another preparation from Dr David Scott, which had a marked glycogenolytic effect. It stood out particularly clearly in perfusion experiments on rabbit livers, because in that case the blood sugar concentration falls steeply. On the addition of Scott's insulin, the decline of the blood sugar concentration turned into an increase. However, the Abel preparation had no effect on the blood sugar curve. For that reason we have relied on the assumption that it was free from the glycogenolytic factor.

Best: I do not know whether it was free, but it was much lower in glucagon

Lundsgaard. Our later experiments were performed with the Novo insulin which C De Duve (6) claimed to be free of the glycogenolytic factor. I do not know whether we could ever say it was completely free.

Best: I think it is virtually free

Lundsgaard. In my laboratory one of my former assistants has carried out some experiments to elucidate the question of whether insulin enhances glycogen deposition in the normal liver. He used the Himsworth technique which enables us suddenly to cut out the liver from the circulation in unanesthetized rabbits Insulin was injected intravenously, and the decline in blood sugar concentration was followed from 20 to 30 minutes Then the liver was tied off and the further decline in blood sugar concentration was followed The aim was to tie off the liver at a stage when hypoglycemia had not yet developed. In the most successful experiments the blood sugar level just after administration of insulin was from 110 to 120 mg. per cent, and the liver was tied off at the level of from 80 to 90 per cent. If the liver takes an appreciable part in the lowering of the blood sugar concentration following administration of insulin, we should expect the decline in blood sugar concentration to slow down after the liver had been cut off. However, such an effect was never observed, and on the contrary, the fall in blood sugar concentration generally became somewhat steeper after the liver had been tied off In some experiments an attempt was made to keep the blood sugar level constant after the injection of insulin [illegible]

[illegible] the blood

Using cats, I have myself repeated some experiments performed on dogs by De Duve *et al* (7) The disappearance of glucose after a supramaximal dose of insulin was measured simply by determining

the rate of glucose infusion necessary to keep the blood sugar level constant In their experiments, the Belgian investigators found that the rate of glucose disappearance in hepatectomized and eviscerated dogs only amounted to one-fifth of the disappearance rate in intact dogs In my experiments on cats I found no difference between normal, hepatectomized and eviscerated animals. According to my results, in cats at any rate, the rapid disappearance of glucose after a large dose of insulin was completely dominated by the uptake in the muscles.

With the experiences gained in my laboratory as a background, I think consideration of the question of whether insulin exerts any direct effect on the deposition of glucose as glycogen in the liver is justified. The unquestionable increase in liver glycogen in a diabetic organism treated with insulin, might in some way or other be secondary to the normalization in the blood sugar level, and the stoppage of glucose loss through the kidneys.

As already mentioned, a rough carbohydrate balance, based on glycogen and blood glucose determination, seems to indicate that a glyconeogenesis is going on in the isolated cat liver. I may, in this connection, mention that the blood lactate concentration rapidly drops to a very low level after the start of such a perfusion experiment and remains very low throughout the experimental period The extent of the glyconeogenesis, however, seems to be very modest. In experiments on livers from cats, starved for several days or kept on a fat diet for about a week, I have found a glucose output from the liver of from 1 to 2 mg per minute. This must be called a modest output when it is taken into consideration that the general metabolism of a cat, weighing 3 kilos, corresponds to the oxidation of about 25 mg. of glucose per minute. It must also be called a modest output when compared with the glucose utilization of the extrahepatic tissues which various investigators have observed, by the simple method of determining the rate of glucose infusion necessary to keep the blood sugar level constant in hepatectomized or eviscerated animals

In experiments of this type on cats I have found a glucose utilization in the periphery of about 7 mg per minute In my experiments, the average blood sugar concentration was 114 mg per cent. This is a high level compared with the blood sugar concentration in a starving animal This also applies to similar determinations performed by other investigators The rate of glucose utilization in the extrahepatic tissues is to a marked degree dependent on the glucose concentration It is likely that the extrahepatic glucose

utilization is definitely lower under such circumstances, where the maintenance of the blood sugar level is solely dependent on a glyconeogenesis in the liver. I do not think that it can be excluded that a glyconeogenesis of from 1 to 2 mg. per minute is sufficient to maintain a blood sugar level of 70 mg per cent in a starving cat. On the other hand, this assumption seems scarcely compatible with the view that the metabolism of the central nervous system is exclusively a carbohydrate one if, as it is now generally stated, the oxygen consumption of the brain amounts to 15 per cent of the total resting oxygen consumption

In my experiments on livers from starved cats, and those fed on fat, the ratio between increase in blood glucose concentration and increase in blood urea nitrogen was determined The dextrose/nitrogen-quotient calculated in this way ranged between 3 and 5. This order of magnitude should be compatible with the assumption that glucose is formed exclusively from protein I am only touching on the classic problem of whether fats in the liver are converted into glucose, which is given off from the liver to the blood I shall not venture further into this much debated classic question, but I may mention that through the years I tend, less and less, to believe that such a net formation of glucose from fats plays a part in the metabolism of the animal organism

Shorr: May I revert to the problem of the source of energy of the brain as it relates to the justification of using total oxygen consumption as a measure of its carbohydrate metabolism? Although we are prone to consider glucose as the sole source of energy for brain metabolism, an assumption which would justify using the measurement of oxygen consumption to estimate glucose oxidation, actually only a portion of the brain, namely the gray matter, utilizes glucose exclusively The white matter, on the other hand, has a low R Q , therefore, the accurate use of the total oxygen consumption of the brain for the calculation of glucose consumption would require that it be corrected for the relative oxygen consumption of gray matter versus white matter.

Lundsgaard But the metabolic rate in the white matter is very low compared with that of the gray matter, and direct determinations, even performed in humans, on the oxygen and carbon dioxide content in arterial and venous blood coming from the brain have, so far as I know, given a quotient of unity.

Best Didn't Himwich (8) obtain a quotient of unity?

Shorr He got a quotient of almost unity However, it is true that if we use only cortex, we shall obtain an R Q of unity. If we include

more and more white matter, the R.Q. falls in proportion to the extent of admixture with it. Certainly, the R.Q. of white matter *in vitro* is not in the neighborhood of 1.

Goldblatt: Might that be an indication of a great difference in the relative amount of blood circulating through these two different parts of the brain?

Shorr: It could easily be.

Lundsgaard: Undoubtedly the blood flow through the two different matters is proportional to their metabolic rate. It is the same thing if we say there is a difference in the blood flow, or in the metabolic rate of the two matters.

Best: The slice technique does not give the same answer as perfusion, in many cases, but I think we should all agree that we prefer the perfusion to the slices, and the whole animal to the perfusion.

Lundsgaard: Yes. Although we have not carried out systematic experiments to investigate the effect of insulin on the protein metabolism of the isolated cat liver, I think it is safe to state that we have never observed any effect of insulin on the urea or glucose output from such a liver. This may be summed up by saying that we have found no evidence of an effect of insulin on the glyconeogenesis apparently going on in the isolated cat liver. This, of course, has strengthened my doubt as to whether insulin exerts any direct effect on the liver.

But the fact that no net carbohydrate metabolism can be demonstrated in the isolated cat liver does not necessarily mean that it does not take place. Livers from normal cats starved only from 24 to 48 hours put out only quite small amounts of ketone bodies, and those from pancreatectomized cats, or from those treated from 2 to 3 days with phlorhizin, put out very large amounts of ketone bodies. Nevertheless, no difference between the two types of liver as to the carbohydrate balance was found. The respiratory quotient of the livers putting out large quantities of ketone bodies was considerably lower than that of the normal livers, but it is a simple consequence of the ketone body formation in which oxygen is used without any simultaneous formation of carbon dioxide. Offhand, one might conclude from these observations that the extent of carbohydrate metabolism was of no consequence for the rate of ketone body formation, which would be contrary to the prevailing view. As I said before, a lack of a net carbohydrate metabolism in both types of livers does not necessarily mean, however, that no

differences exist in the metabolism of carbohydrates or the carbohydrate turnover in the two types of livers

Gurin: Dr. Lundsgaard, I think it is rather interesting that the liver in the previously fasted cat is still able to synthesize fat, whereas the liver in the previously fasted rat could not. I have not worked with a large number of species, but we were rather sur- [illegible]

in connection with what you say.

Lundsgaard. Yes, certainly. During the last year, Dr. J A. Muntz (9), of the Biochemistry Department of Western Reserve University, has been working in my laboratory and has carried out experiments on isolated cat livers using glucose labeled with C^{14}. I shall not go into details as to the technique employed, but I shall mention one complication. As the isolated cat liver metabolizes lactic acid and may store lactic acid as glycogen, in Dr. Muntz' experiments it was necessary to prevent glycolysis of the plasma glucose. This was done by poisoning the red corpuscles with iodoacetate, washing them lightly and suspending them in the original plasma.

Dr Muntz' experiments have revealed that an oxidation of carbohydrate actually takes place in the isolated liver of a cat. Judging by the activity in the metabolic carbon dioxide, the carbohydrate oxidized only covers from 3 to 5 per cent of the total oxidative metabolism of the liver. This figure, however, is a minimum figure, as it is calculated on the basis of the activity of the plasma glucose. Dr Muntz' experiments also revealed that plasma glucose is built into liver glycogen at a not inconsiderable rate The disappearance of activity from the plasma could not be accounted for by the activity found in carbon dioxide and glycogen. The reason for this is probably that plasma glucose enters the pool of phosphate esters of the liver

Unfortunately, only a few experiments on livers from cats poisoned with phlorhizin have been performed, the results did not differ in principle from the experiments made on normal cat livers Also, in the phlorhizin livers, an oxidation of carbohydrate of the same order of magnitude as in the normal livers could be demonstrated. However, the experiments are too few to decide whether any difference exists between the two types of livers with regard to the rates with which plasma glucose is incorporated into liver glycogen and enters into the pool of phosphate esters

Personally, I think it inconceivable that an exchange of glucose

molecules between plasma and liver glycogen could be a consequence of ketogenesis. It is more difficult to conceive of the significance of the entrance of glucose from the plasma into the pool of liver phosphate esters. If this entrance only signifies a simple exchange of hexose molecules, the rate of exchange can scarcely be of any significance in the rate of ketogenesis Only an oxidative degradation of hexoses with a formation of pyruvic acid should be expected to enhance the oxidation of acetic acid in the tricarboxylic cycle and thus counteract a condensation of acetic acid to ketone bodies. With regard to carbohydrate oxidation, Dr Muntz' experiments revealed no difference between normal livers with a small output of ketone bodies, and livers from phlorhizin-poisoned cats with a large output of ketone bodies. The experiments, however, are so few that I do not consider the possibility ruled out that some difference in carbohydrate metabolism between the two types of livers exists which may have some connection with the difference in rate of ketone body formation. Even if we assume this to be the case, some difficulties are nevertheless encountered if one tries to explain the large ketone body formation in the phlorhizin livers simply as due to a blocking of the tricarboxylic cycle.

I think it can be taken for granted that the oxidative metabolism of both types of livers is predominantly a combustion of fats The protein metabolism of livers from depancreatectomized cats and cats poisoned with phlorhizin is slightly higher than in livers from normal cats

Shorr: May I ask what the glycogen content of the 24- to 48-hour-fasted cat is compared to the phlorhizined cat?

Lundsgaard. The liver from the cats starved from 24 to 48 hours is generally in the order of magnitude of 0.1 per cent and in the phlorhizined cat it may go down to a fraction of this, such as 0 02 per cent.

Shorr. You do not think that may have a relationship to the difference in ketone body formation?

Lundsgaard: It may have. If so, the presence of glycogen should account for the difference.

Shorr. On that basis?

Lundsgaard. Until recently I have emphasized that there should be no difference in carbohydrate metabolism, but the difference in glycogen concentration in the two types of livers should be of significance. However, we have now seen that there is a certain turnover in carbohydrate, even in the cat livers, where there is no net or over-all carbohydrate metabolism I think one has to look

for a difference in this turnover. So far we have not been able to demonstrate any difference, but I do not dare to rule out the possibility completely.

I wish to emphasize that even if we assume such a slight difference, which so far we have not been able to demonstrate, there are some difficulties in explaining the difference in the metabolism in these two types of livers.

Gurin Would that not be involved in the metabolic fate of acetyl coenzyme A, a two-carbon fragment which is undoubtedly the source of the ketone bodies? You referred to the oxidative fate of acetyl coenzyme A indirectly, and I assume that is what you were talking about. There are a number of metabolic fates that may be considered with respect to what happens to acetyl coenzyme A, its conversion to acetoacetyl coenzyme A, which is undoubtedly the immediate precursor of ketone bodies There is an enzyme involved in the hydrolysis of acetoacetyl coenzyme A Should this become inactivated in one case, fewer ketone bodies would be formed. The acetyl coenzyme A can be used for resynthesis of fat, which brings up the question of the liver of the phlorhizined animal being able to resynthesize fat, or utilizing coenzyme A for synthesis or other assimilations, and so on I think this is a rather difficult question to answer at the moment

Lundsgaard I cannot tell whether these livers are able to synthesize fatty acids There is a very pronounced breakdown of fat going on in these livers

Shorr. Actually, then, the situation is this: although once the liver is out of a phlorhizined or normal animal, you cannot demonstrate any net gain of carbohydrate, yet ketone body formation is strikingly different, and the only correlation that is conceivable is the "set" of the animal at the time you take the liver out, at which time the normal animal has more carbohydrate than the phlorhizined one.

Lundsgaard Yes, that is just what I am going to bring out. I use the expression, "the setting" As protein metabolism constitutes only a minor fraction of the general metabolism of the liver, and as no net carbohydrate oxidation is demonstrable in the liver, I think one may take the carbon dioxide output as a rough measure of the complete oxidation of fats in both types of livers, whether they have small or high ketone body formation As the fatty acids constitute 90 per cent of neutral fats, this again means that the carbon dioxide output can be taken as a measure for the complete oxidation of fatty acids Now, the carbon dioxide output from both types of

livers is of the same order of magnitude. Apparently the tricarboxylic cycle is not blocked in a liver putting out large quantities of ketone bodies, as such a liver oxidizes fatty acids completely at the same rate as does a normal liver. The tricarboxylic cycle might nevertheless be blocked. Then one has to assume that the last steps in the oxidation of fatty acids in a liver putting out large quantities of ketone bodies do not pass through the tricarboxylic cycle, but consist in an oxidation of ketone bodies following a different route.

Even if one makes these assumptions, it is, I think, difficult to conceive how such a change in the last steps in the oxidation of fatty acids can directly influence the rate of the first steps in the oxidation of fatty acids, which are increased in a liver putting out large amounts of ketone bodies. The rate of the total fatty acid metabolism, as measured by the output of ketone bodies plus carbon dioxide output, may be four to five times as high in a liver from a cat poisoned with phlorhizin, as in a normal liver.

It is my experience that it is impossible to change the type of metabolism of an isolated liver. The liver, when isolated, is either "set" for a vivid metabolism of fatty acids with formation of large quantities of ketone bodies, or for a considerably less vivid fat [illegible] t [illegible] ces should then [illegible] the fat metabolism. However, the different "setting" might also be due to differences in the hormonal "setting" provoked by the impairment of the carbohydrate metabolism of the organism as a whole.

Shorr: Have you ever analyzed the whole system? In perfusion experiments one has to assume that what one is determining by analysis of the blood is a complete reflection of what is going on in the tissue. This need not be the case. I wonder whether you have obtained a complete balance sheet in any of the experiments which include both the perfusion fluid and the liver. This may be important because certain reactions may go through more than one pathway, for example, in the liver acetate may either be oxidized through the tricarboxylic acid cycle, or accumulate in excess as acetoacetic acid [illegible] tion as to which [illegible] there is an accum[illegible] may require the use of certain enzyme inhibitors such as fluoroace-

tate, which make it possible to determine the magnitude of acetoacetate formation by preventing it from going through the tricarboxylic acid cycle Is my reasoning wrong about this possibility?

Gurin: No, I do not think you are wrong. It seems to me that there is an increasing body of evidence that liver slices, or liver tissue taken from experimental diabetic animals, are capable of handling or oxidizing lactate, decarboxylating pyruvate, forming acetyl fragments, and oxidizing acetate via the tricarboxylic cycle. I do not see how it is possible, at the present time, to make much of a distinction between fat and carbohydrate oxidation So far as the whole balance is concerned, I can understand that if we are driving a series of reactions in the direction of glucose, which is excreted from the whole animal, we are forcing an equilibrium in one direction or the other. However, it seems to me that it is worth while considering the fact that the enzymes for oxidation of either carbohydrate or fat are essentially intact By carbohydrate, I mean, of course, the metabolic products such as pyruvate or lactate which can be derived from glucose If the glucose in the diabetic state cannot be phosphorylated, obviously carbohydrates cannot be oxidized However, so far as the major metabolic products which are present in the tissue are concerned, it seems to me that in the diabetic state we have the enzymes, the total mechanism there, which are able to oxidize them both Since both fat and carbohydrate can give rise to the same common intermediate, acetyl coenzyme A, so far as the oxidation is concerned, I do not see that we can make any distinction between the two major foodstuffs, fat and carbohydrates But perhaps this is a one-sided biochemist's point of view

Best I do not know whether we have enough information to debate the significance of the findings in the liver slices.

Gurin Let us go back to the whole animal.

Best It does seem to be a little easier to influence the slice than it is to influence the perfused liver. We have to give some things to the animal to obtain the effect on the slice, but we can add the insulin, say, to the slice and get glycogen formation Is that not so?

Lundsgaard That is the reason why I think it is really justifiable to discuss whether insulin has a direct effect on the liver. Offhand you might think you would be able to answer this question; in the whole animal, of course, insulin has an effect on the liver. However, I am not convinced that it has ever been proved that insulin has a direct effect on liver tissue.

Gurin One can demonstrate, I think, a number of minor defects

of *in vitro* effects of insulin. This bears out the point that Dr. Best made. For example, the incorporation of labeled acetate and pyruvate into fat is stimulated in normal liver slices by the addition of insulin This has been done in several laboratories Just what this means, and whether or not it is important, I am not prepared to say. My own feeling has been that this is some sort of secondary effect which makes available a little more of the energy required for lipogenesis One can demonstrate this in normal animals, but not in diabetic animals.

Shorr: Dr. Gurin, it has been stated that in the diabetic rat, the turnover is profoundly reduced, not only in the carbohydrate cycle, but even more so in the fatty acid pool (10). If we have a profound reduction in fatty acid turnover, and at the same time a markedly increased output of ketone bodies, then the bottleneck might be the passage of the two carbon fragments through the tricarboxylic cycle The bottleneck may be coenzyme A. What is your opinion?

Gurin: I should like to reserve that for a little later.

Fremont-Smith. You see, this is very interesting It is the sort of false position we get into when we try to have a discussion of carbohydrate metabolism and then later, a discussion of lipoid metabolism. Really, one should have a discussion of the two together

Gurin. I think proteins could well come into this, too.

Fremont-Smith I did not mean to eliminate the proteins

Best In our recent deliberations on the future of physiology, I think we agreed that it would be most profitable to have the organic chemists do whatever they like on small bits they can get from the body, such as fractions of cells, and so on, and the biophysicists do what they like, but there will probably always be a place for what we would call integrative physiology, because, after all, we do want to know how all these things are coordinated and how they function in the intact organism

Gurin: I think that is what we are interested in, too, Dr Best.

Best At the same time, physiology should never inhibit the organic chemist from going as far as he likes, so long as he helps us to arrive at a better understanding of the whole picture

Gurin Exactly I think this is really the motivation behind our own work and that of many others who are interested in the intimate mechanisms involved: to discover what actual steps, chemical, metabolic, or enzymatic, are involved in this tremendously complicated series of reactions Once some of these steps have been worked out, they have to be integrated into the behavior of the

whole organism I think this is much more difficult than what we are doing.

Best· There might be a chemist working on an enzyme, and another working on an antienzyme, but if they were put together, nothing at all would happen

Fremont-Smith· Another way to say it is that the chemist tries to specify all the possible reactions, and then, when we reach the point of reintegration, we have to try to find out which are the actual reactions and which have the highest priorities under different circumstances. However, until we know all the possible reactions of the isolated fragments, we are not in a position to judge which one might be selected in the more integrated organism

Shorr It is surprising that the pathologists have not asked what the liver cell looked like at the end of two hours of perfusion

Best It looks worse and worse, does it not?

Lundsgaard: I never had a pathologist look at the livers I was working on; perhaps I should have

Best: From my own experience, I can tell you that it is very disturbing to have a pathologist look at the perfused liver. However, it is obviously one step nearer the intact organism than the liver slice

Later on I hope our clinical friends will discuss abnormalities in carbohydrate metabolism in the liver I do not mean the diabetic state, but cirrhosis, galactosemia, the pathology in von Gierke's disease, or something of that kind I think it would make it a better record if we had here some comments from the pathologist or clinician about morphological changes associated with interferences with carbohydrate metabolism.

Fremont-Smith May I go back to a point which arose from the suggestion that we look at the histopathology of a perfused organ and a perfused liver, and the progressive changes that take place? Would it not be interesting and perhaps revealing to make liver slices at different times successively in such a perfused liver, and see how they behaved at the end of the experiment? Might you not be able in this way, to get a picture from a different angle of some of the changes, or lack of changes, in the fundamental processes going on in such a perfused organ?

Lundsgaard There may, of course, be some function which you can study more easily with the slice technique, but changes in the different reactions in the liver should stand out, too, in the entire organ The rate of the general metabolism as measured by the oxygen consumption in such an isolated liver, is undoubtedly lower

than normal. We always see that at the start of such a perfusion, the rate of oxygen consumption is fairly high, but then, in half an hour, it drops, and finally reaches a rather steady level without any further decline.

Best: It is obviously an indication of our ignorance that we obtain pathological changes in the perfused liver. If we knew how to perfuse the liver, there would not be any. It should look normal at the end of the time.

Fremont-Smith: If certain reactions in the liver cells were the same in the middle and at the end of the perfusion experiment, it would have a more definite significance than if there were gross differences in the behavior of a liver slice at the beginning and end. and this would provide a kind of secondary control. Is that a possibility?

Lundsgaard· Oh, certainly.

Watson: Dr Best, I was wondering about the rate of bile formation and the constituents of the bile Did you study this at all during the two-hour period of perfusion?

Lundsgaard. No, we have never been interested in the study of the bile.

Watson: I wonder if you could use that as a measure of normality?

Lundsgaard. Yes, I believe one could.

Watson You would obtain a group of curves, and after a certain period of time they would all start to fall off at different rates. As long as they were on a level, I imagine that that would give you some index of the function of the perfused liver.

Lundsgaard: Of course, it would be quite elucidative to measure the rate of bile formation, but we have never done it.

Shorr: Or bromsulphalein clearance?

Best Is there a steady bile formation?

Lundsgaard: I cannot say

Watson: If the pigment and bile salt were quantitated, I think those two things, together with the amount of bile, might give us a pretty fair insight into what the liver was doing.

Best That is a perfectly simple, direct, obvious problem: to perfuse a liver so that bile formation remains normal, so that it stores glycogen when we wish it to, as in the intact animal. Yet, no laboratory has been able to do that.

Kosterlitz I wonder whether Dr Lundsgaard would be prepared to enlarge a little on why glyconeogenesis in the perfused liver is not as large as would be expected from theoretical con-

siderations. You said, Dr. Lundsgaard, that the amount of glucose formed is below requirements. Have you any views on that?

Lundsgaard: Yes. What I intended to say was that the output of glucose which I have found in the isolated liver is modest, and if it is compared with the figures from the literature for the utilization of glucose in the periphery, it seems too low to maintain the blood sugar level. According to my own experiments, in which the peripheral utilization was determined simply by keeping up the blood sugar level by infusing glucose at a constant rate, the output was also low. I found a utilization of about 7 mg. per minute, and an output from isolated livers of from 1 to 2 mg per minute. However, I then pointed out that the blood sugar levels at which the determinations of the peripheral utilization had been made, in my own experiments, and those of most others, was rather high, and the utilization in the periphery was markedly dependent on the concentration For that reason I would not dare say that this output of from 1 to 2 mg per minute was too low a figure to keep up a blood sugar concentration of, say, 70 mg per cent, which would be found in the fasting animal That is one point which might be discussed along with the question of the rate of glyconeogenesis in the liver, because there is some discrepancy there

When I saw this rather small output of glucose from the liver, I performed some experiments in which the blood, which was taken up by the pump in my perfusion experiments, was divided into two streams· one going through an isolated liver, and one pumped into the jugular vein of a hepatectomized or eviscerated cat At the same time, an equal amount of blood was taken from the carotid artery of the cat and added to the perfusion system, so that the liver was connected with a hepatectomized animal I first ran the liver for three-quarters of an hour, which was a sufficiently long period to determine the glucose output from the liver.

I found a considerable increase in glucose output from the liver after it had been connected with the hepatectomized cat, but it turned out that this was simply due to the rather high blood lactate concentration one obtained when the hepatectomized cat was connected with the system One could demonstrat [illegible]

[illegible]

situation of what is generally called a Cori cycle, and it was not really an effect on a true glyconeogenesis

Kosterlitz I was wondering whether the addition of adrenal corticoids to the perfused liver would influence glyconeogenesis.

Shorr: May we add epinephrine to that?

Kosterlitz: Yes.

Lundsgaard. We have performed some experiments with cortex hormones, but I have not seen any effect. I think our experimental period is too short; the effect is not so rapid that it may be observed in a two-hour period.

Shorr: If the presence of epinephrine is so critical a determinant of the rate of glycogen breakdown to glucose, would the epinephrine content of the perfusion fluid be important to consider in relation to the results? Do you think there is much epinephrine left in the perfusion fluid?

Lundsgaard: No.

Shorr: Hence, one of the important determining factors in the release of glucose from the liver is absent.

Lundsgaard: Yes

Shorr: Perhaps the low value may be in part a reflection of the absence of epinephrine from the perfusion fluid?

Lundsgaard: That is unlikely, because in the experiments in which we determined the glucose output from the liver, the glycogen content of the liver was so low that a slight change in the percentage of liver glycogen was of no consequence in the balance. We cannot rely on the glucose output if there is a fairly high glycogen content in the liver, then it is impossible to say anything about the glyconeogenesis. Glycogen content in the liver, a slight breakdown of glycogen, or a slight increase of glycogen, would all upset the calculation of the glyconeogenesis.

Shorr: Thus, if the liver perfusion were not an ideal perfusion, artefactual glycogen breakdown would produce an artefact in terms of glyconeogenesis

Lundsgaard: Yes. I should also say a deposition of glycogen Of course, glycogen content cannot be determined with complete accuracy Therefore, if we have a fairly high concentration of glycogen in the liver, slight differences from the beginning to the end of the experiment would influence the calculated glyconeogenesis to such an extent that we could not rely on the result. However, if we have a very low glycogen content at the start, as well as at the end of the experiment, then I think it is safe to use the actual changes in the blood sugar concentration as a measure of the glyconeogenesis

Shorr Of course, in that case the assumption is that the only source of glucose in the perfusion fluid coming out of it is the glycogen, and protein is not a contributing factor.

Best. In a short experiment?

Lundsgaard: I am afraid you have not quite understood me. Glyconeogenesis, as I use the term, is the formation of carbohydrates, or glucose, from other sources than glycogen and lactic acid In the liver preparation, the lactic acid concentration is extremely low and keeps low, and if there is a very low concentration of glycogen in the liver at the beginning and the end of the experiment, we can be sure that glycogen breakdown, or the deposition of glycogen, has not influenced the glucose concentration in the blood We can then take the changes in the glucose concentration in the blood as a measure of glyconeogenesis

Shorr. My original question referred to the possible influence of the epinephrine content of the perfusion fluid on the rate at which glucose is provided to the fluid by the liver That is, the levels were so low as not to equate the energy consumption with the amount of glucose used by the perfused muscle I also wondered whether the low values for glucose in the blood might conceivably have an explanation in the absence from the perfusion fluid of one of the factors that ordinarily leads to an increase of glucose production by the liver

Lundsgaard Do you think that epinephrine leads to an increased production of glucose by any other means than by the breaking down of glycogen?

Shorr No, only through carbohydrate breakdown However, whatever the cause, it is not in the perfusion fluid

Lundsgaard No but practically if there is no glycogen in the liver, as is the case when an animal has been starved for a long period or kept on a fat diet for about a week, we should expect the glyconeogenesis to proceed at a high rate

Shorr I should regard protein as a potential source of the glucose

Lundsgaard Yes, certainly

Shorr That might be influenced by epinephrine just as is the case when glucose is derived from glycogen

Lundsgaard Would glyconeogenesis from protein be influenced by epinephrine?

Shorr I should think that it might We know there is a profound increase in the glucose excretion by the phlorhizined and diabetic animals who have lost their liver glycogen and are receiving only protein in their diet Since glycogen cannot be the source of the glucose it is generally assumed to come from protein If we could transfer these in vivo observations to the perfused animal, then I

should think this process would also be seen under the conditions of the perfusion.

Best: However, in the intact animal we have the indirect effects of epinephrine in the liberation of anterior pituitary material

Shorr: That is one possibility which we cannot exclude. My point is that we do not seem to have enough data to draw up a complete balance sheet, and it was my thought that humoral factors may be responsible for this discrepancy.

Fremont-Smith: I am more and more convinced of the necessity of using different techniques in the same laboratory on an experiment. I think the tendency to isolate a special technique and develop it to a greater and greater degree of refinement, sometimes blinds us to the possible advantage of simultaneously doing something else in the same experimental procedure that would give us another cross-sectional view of what is going on.

Best: I agree that it would be interesting if Dr. Lundsgaard had the facilities to study three preparations instead of one: (*a*) a liver slice from an animal prepared from the dietary and hormonal point of view, (*b*) liver slices from the same animal, or a comparable one, and perfused by the method he has been using; and (*c*) the intact animal with the circulation arranged so that he could measure and sample the ingoing and outgoing blood from the liver If that were done under as nearly comparable conditions as possible, I am sure he would get widely divergent results and have a great deal of difficulty in integrating the answers. As it is now, Dr. Lundsgaard can only do one. Some other laboratory works under conditions that are not comparable, and we struggle to coordinate the results. It is a hard way. We make it difficult for ourselves.

Shorr: Dr. Soskin (11) did that type of experiment *in vivo* with low and high levels and found that whenever the arterial levels of glucose were high, the hepatic vein blood was lower; whenever they were low, the hepatic vein blood contained a higher concentration

Best. Dr. Soskin really interpreted his answers on the basis of the concentration of sugar, but he was also influencing many other things when he raised the level of sugar. I mean, the hormonal balance could have changed by the level of sugar which he maintained.

Fremont-Smith: When a coordinated, simultaneous attack by means of several different techniques in the same laboratory is indicated, would it be possible to organize a temporary cooperative effort? It is usually not possible for one laboratory to provide facil-

ties for four or five different techniques at once, but at a crucial time, when a certain question needs to be answered, might it not be possible for a group of people to work together for six months in Dr. Lundsgaard's laboratory, for example, or in Dr. Best's laboratory, or omewhere else, and after the work has been completed to return) their own laboratories again? This would be a cooperative rearch by mature investigators, each having a different slant and chnique. Is this an idea that we should think about seriously?

Best It would be very expensive, I suppose, but considered in terms of results it might be economical It can and is being accomplished For example, Dr Houssay works at Bethesda for a while with people there who have techniques other than his So, in a limited way, this is now being done, but not in a well-planned or comprehensive way Dr Houssay would have liked to bring several other people from his group

Houssay Dr Best, perhaps what we need are not only more facilities for work, but more ways of developing new ideas and obtaining new facts

Best I think this method of approach would encourage the development of ideas

Fremont-Smith: For instance, in the kind of experiment Dr Best suggests, there would either be a concordance, which would provide a basis from which progress could be made, or else, more likely, there would be discrepancies which could not be understood, showing that none of the concepts arising from any of the various methods would hold and that something was missing which should be searched for

Best Coordination of work should be extended and better organized there is no doubt about that

Fremont-Smith It does seem to me that one of the things we now lack is the cooperative research of mature investigators who have different backgrounds and different approaches to a common problem Very often, we have young men going to older men, but we do not have mature investigators coming together and saying Now let us see if we cannot settle this issue which could perhaps be illuminated by two techniques'

Best Some of us go to other laboratories and lecture, but we get very lonesome We miss the assistants in our own laboratories

Fremont Smith One would have to move a team perhaps take three people from one laboratory and set them up in a host laboratory However there would have to be a real issue, worth working on

Best: Those of us who have spent time perfusing organs might do it better. We might acquire more of the existing knowledge, or new knowledge that might help us. However, it is interesting to reflect that from the perfusion of the heart, which Dr. Otto Loewi (12) carried out – a rather unimportant one from the physiological point of view – he discovered the liberation of acetylcholine, one of the greatest discoveries of our time.

Shorr. From a frog!

Best: It is really the result obtained, and not the perfection of the technique, that matters I am now going to ask Dr. Houssay to speak

Houssay: The liver plays an important role in the production of glucose, in the homeostasis of the blood sugar level, and in the regulation of carbohydrate metabolism. The classical experiments of Mann, Magath, and Bollman, which many of us here have repeated and confirmed, has shown that the liver is the principal site of the formation and release of glucose in a quantity adequate for the maintenance of normal glycemia. After hepatectomy, the blood sugar falls, and it is impossible to produce a hyperglycemia, either of diabetic or other origin.

The liver is able to form and store glycogen and use it for the formation of glucose, and is also an important organ for the formation of fat from carbohydrate. It is not possible to study carbohydrate metabolism of the liver without considering that this metabolism is connected with fat and protein metabolism, the phenomena of fat and protein metabolism are dependent on carbohydrate metabolism

For the study of the role of the liver in carbohydrate metabolism, many methods have been used: For instance, in the whole animal we may observe the balance between the glucose which enters and leaves the liver. The extirpation of the liver has also been a very

first, to begin with the whole animal, second, to try to analyze the biochemical and biophysical functions by all the means I have mentioned, and third, to come back again to the whole animal, because physiology represents integration of knowledge. We wish to know what the role of the function of the liver is in the integration of the functions of the whole organism

A discussion of the problem of carbohydrate metabolism and the liver is provisional, because we have only a very general knowledge and have many things to learn It is useful to have these meetings It is a way of discovering our limitations and of learning new ideas or facts with which we perhaps organize in the way that Dr. Fremont-Smith has invited us to work

Best I think it might be interesting to have a brief summary of Professor Hoet's recent results. He does not say much about the liver, Dr Fremont-Smith, but perhaps at a certain phase of development the placenta performs some of the functions of the liver

*Hoet** On the whole, I think this question of glycogen in the placenta more or less covers the liver function, as I said earlier, because the liver plays a role in the nutrition of the fetus only in the last days or weeks of pregnancy, depending on the species

The problem has its place between the preferential drawing of nutritional substances for the different organs, i.e., muscles, brain, liver, skin, and eventually placenta, and the shifting of the priority of those organs at different physiological moments In clinical diabetes, the other side of the problem is seen in the fact that even twenty years before permanent and severe hyperglycemia and loss of tolerance of dextrose, the diabetic woman has a severe pathology when pregnant As a rule she eventually increases in weight more than another pregnant woman, and as a rule she has lost her babies in the past The glycosuria, hyperglycemia, and the permanent disturbance in tolerance to dextrose, will come five, ten, or fifteen years later

In experimental animals we are able to demonstrate first, that there is a very clear-cut diabetogenic effect of pregnancy, and second that the nutrition of the fetus is very sensitive to disturbances of carbohydrate metabolism in pregnancy

Figure 1 shows how we can study the condition of prediabetes in animals We inject 75 mg of alloxan per kilo into a rabbit Curve I is the blood sugar curve after injecting 1 gm of dextrose per kilo

* The investigations reported here were sponsored by the Fonds National de la Recherche Scientifique under a special grant for research on the hormones of the adrenal glands and their physiological control

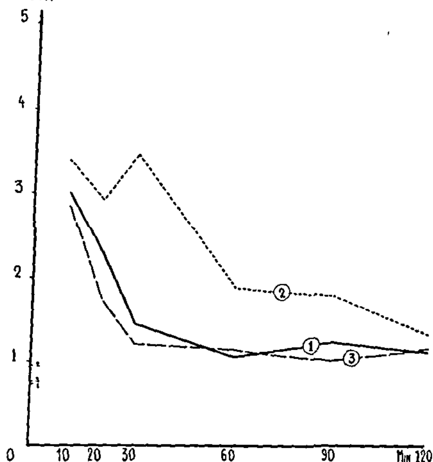


FIGURE 1 R 151 Alloxan 75 mg per kilo Becomes pregnant 9 days later (1) 9 days after alloxan (1st day of pregnancy) (2) On the 15th day of pregnancy (complete resorption occurs on the following days) (3) After resorption

During pregnancy, which takes place fifteen years before she becomes permanently diabetic, she is a transitory diabetic from the third or fourth month until the birth of the child

This problem has been studied by Barns and Reid (13) and also by Young (11), from the point of view of growth hormone. During pregnancy, if small amounts of growth hormone are injected, we see that the animal does not become diabetic, and second, although the fetuses die off, there is no transitory glycosuria

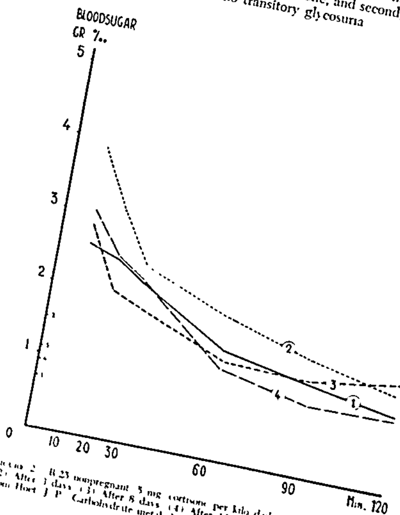


FIGURE 2 B 23 nonpregnant 5 mg cortisone per kilo daily (1) Before cortisone (2) After 3 days (3) After 8 days (4) After 13 days Reprinted by permission from Hoet J P Carbohydrate metabolism during pregnancy *Diabetes* 3, 1 (1954)

Our results on the action of the glucocorticoids seem to cover the clinical field much better, because as you know Venning and Browne (15), Nelson and Samuels (16), Bush (17) and others, showed that pregnancy is a physiological Cushing syndrome from the hormonal point of view, and that is what we shall see now

In Figure 2, we observe that when we inject 5 mg per kilo of cortisone into an animal which is not pregnant, we do not see any difference in the blood sugar curve. When we inject 2 mg. of

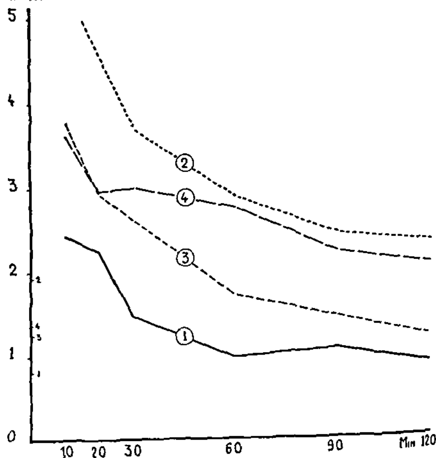


FIGURE 3 R 79 pregnant. 2 mg hydrocortisone per kilo daily from the 12th day forth (1) Before pregnancy (2) On the 15th day. (3) On the 18th day (resorption) (4) On the 21st day. Reprinted, by permission, from Hoet, J P. Carbohydrate metabolism during pregr[illegible] [illegible]etes 3, 1 ([illegible])

hydrocortisone into an animal which is pregnant (Figure 3), we see, in curves 2, 3 and 4, in three days' time the animal develops diabetic hyperglycemia. At the 18-day level, under hydrocortisone, the resorption of the fetuses takes place.

Figure 4 shows that if we give ACTH during the pregnancy, the same thing occurs. The pregnant animal is very sensitive to the disturbing action of glucocorticoids on carbohydrate metabolism, and with 2 mg. per kilo, we see the blood sugar curve going higher,

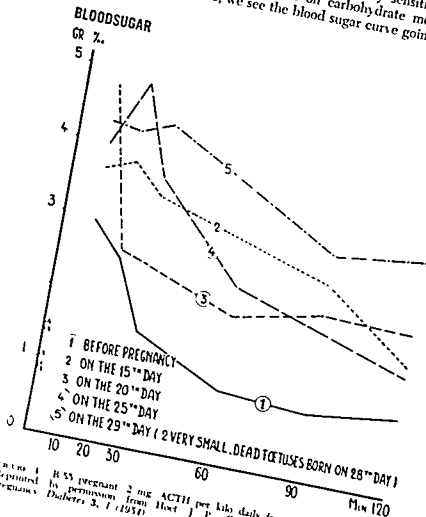


FIGURE 4. R 55 pregnant. 2 mg. ACTH per kilo daily from the 12th day forth. Reprinted by permission from Hoet, J. P. Carbohydrate metabolism during pregnancy. Diabetes 3, 1 (1954)

and at the end, the fetuses are dead That animal, a few days after the death of the fetuses, becomes normal again, from the sugar tolerance point of view.

To put it briefly, the nutrition of the fetus is highly disturbed by doses of 2 mg. of cortisone or ACTH per kilo. Therefore, we went down to a dosage of 0 5 mg. cortisone per kilo body weight of animal, which does not kill the uterine contents. With that dosage we were able to study the action of the glucocorticoids, which increased, physiologically, on the glycogen in the placenta.

In 1908, in the laboratory of Sharpey-Schaefer, Lochhead and Cramer (18) studied glycogen in the placenta. The first statements of Claude Bernard were published in 1853

To study glycogen in the placenta, we must realize the difference between two sides of the placenta. the maternal side, which we shall call the "maternal placenta," and the fetal side, which can be decorticated.

My son Peter and I (19) have been able to show that 0 5 mg of cortisone, given for 3 or 4 days, increases very markedly the glycogen in the placenta, and more so on the fetal side.

TABLE I

Glycogen Percentage in Rabbit's Placenta on 14th Day of Pregnancy After Four Daily Doses of Cortisone (0.5 mg. per kilo)

Maternal Placenta		Fetal Placenta	
Control	Cortisone	Control	Cortisone
2 01	5 25	—	1 45
3 60	5 78	0 52	1 48
4 10	6 24	0 64	2.70
2 00	4 70	0 64	2 50
1 80		0 57	1.85

Reprinted, by permission, ... J. P Carbo ... abolism during pregnancy Diabetes 3, 1 (

I shows the d ... from tw
. Since the ... cogen i ... nated at the
to three day ... ancy ... nta chan es
e. The co ... not ... them
nc, l

experimental one received 0.5 mg. per kilo of cortisone from the tenth day onward. We see that cortisone gives an increase in the glycogen. There are five figures, which represent five placentae of the same animal, and we see that the increase is very plain. However, more significant still are the happenings on the fetal side of the placenta, we see that the increase is higher there than on the maternal side. This means that nutrients, carbohydrates, are turning over at a quicker rate to the fetal economy. Later on, this may increase the weight of the fetus. We have just one experiment in which we think we have demonstrated that point. Our evidence is insufficient. However, the increase of nutrients going over to the fetal side of the placenta is a forerunner of the increase in weight of the fetuses.

Fremont-Smith: Professor Hoet, why do you say that there is more on the fetal than on the maternal side? It looks to me as though it were the other way around.

Hoet: Because one day or two days later, without cortisone, that figure of 5 gm. of glycogen per 100 gm. of placenta may be reached physiologically even without cortisone. The figure which we obtain on the fetal side is higher than can ever be obtained in any animal if no cortisone whatever is given. Therefore, the figures for the fetal placenta have a greater demonstrative value than the ones for the maternal placenta.

TABLE II

Newborn of Prediabetic or Diabetic Mothers

- Excessive Body Weight
- Macrosomia
- Hyperplasia of the Islets of Langerhans
- Cardiac Hypertrophy or Congenital Defects
- Erythroblastosis
- Hepatomegaly

Table II shows data on the offspring of prediabetic mothers, which do not include treated diabetic mothers. The table lists, first, excessive body weight; second, macrosomia; third, hyperplasia of the islets of Langerhans, which is very important and is the result of the malnutrition of the fetus during pregnancy — Van Beek (20).

and at the end, the fetuses are dead That animal, a few days after the death of the fetuses, becomes normal again, from the sugar tolerance point of view.

To put it briefly, *the nutrition of the fetus is highly disturbed by doses of 2 mg. of cortisone or ACTH per kilo* Therefore, we went down to a dosage of 0.5 mg. cortisone per kilo body weight of animal, which does not kill the uterine contents. With that dosage we were able to study the action of the glucocorticoids, which increased, physiologically, on the glycogen in the placenta

In 1908, in the laboratory of Sharpey-Schaefer, Lochhead and Cramer (18) studied glycogen in the placenta. The first statements of Claude Bernard were published in 1853.

To study glycogen in the placenta, we must realize the difference between two sides of the placenta: the maternal side, which we shall call the "maternal placenta," and the fetal side, which can be decorticated

My son Peter and I (19) have been able to show that 0 5 mg of cortisone, given for 3 or 4 days, *increases very markedly the glycogen in the placenta, and more so on the fetal side.*

TABLE I

Glycogen Percentage in Rabbit's Placenta on 14th Day of Pregnancy After Four Daily Doses of Cortisone (0.5 mg. per kilo)

Maternal Placenta		Fetal Placenta	
Control	*Cortisone*	*Control*	*Cortisone*
2 01	5 25	–	1 45
3 60	5 78	0 52	1.48
4 10	6 24	0 64	2.70
2 00	4 70	0 64	2.50
1 80	4 98	0 57	1 85

Reprinted, by permission, from Hoet, J P Carbohydrate metabolism during pregnancy *Diabetes 3, 1 (1954)*

Table I shows the data obtained from two animals, mated at the same hour Since *the value of glycogen* in the placenta changes every two to three days during pregnancy, we took them just at the same time The control animal did not get cortisone, but the



FIGURE 5

of Leiden, has traced it for more than ten years now, fourth, erythroblastosis; and fifth, hepatomegaly and cardiomegaly, as Miller (21) of Kansas City has shown.

Neefe: What is the physiologic nature of the hepatomegaly?

Hoet: I am not sure, but it is highly probable that it is due to the large amount of glycogen and a very great absolute increase.

Popper: Fat seems to be also present in addition to much glycogen in the liver of the fetus of diabetic mothers. Is the erythroblastosis comparable to that from rhesus incompatibility?

Hoet: Perhaps the illustration will help to answer that question On the left, in Figure 5, we have a baby born in the eighth month of pregnancy of a prediabetic mother, and we see that it is really blue or bluish red. On the right is a normal one, which is just a term baby, and does not have that Cushing-like face of the baby of a prediabetic, or treated diabetic, mother. A diabetic mother is not going to have a living child without treatment; the nutrition of the fetus will not be sufficient However, a treated diabetic woman will have a child like the one we see on the left. We can observe how bluish they are, they have erythroblastosis.

However, the difference between erythroblastosis and rhesus incompatibility, is that the erythroblastosis fades away the first week after birth, whereas the rhesus incompatibility increases enormously and produces jaundice The children may be absolutely jaundiced, and the liver is certainly quite abnormal. However, it does not persist very long They are passing through a period in which they are in danger of anoxemia We do not know exactly what happens, however, a few die. The question of the hyaline membrane in the lungs plays quite a role in the death of those children They are very hypoglycemic, but it is not the hypoglycemia that will be the cause of death

Figure 6 shows that in the pancreas of children with such an intrauterine malnutrition, the islets are very abnormal That was seen by the embryologist Dubreuil (22), in 1920, and up to 1938 he studied the phenomenon in women and in dogs after subtotal pancreatectomy He obtained an enormous increase in the surface of the islets of Langerhans which we see drawn there.

The relation of that particular kind of pathological pancreas with full diabetes is seen very plainly in the history of diabetic patients, and in Figure 7 we see a patient, Mrs V, who came to us because she was losing her babies However, the characteristic of the stillbirths was that they were heavy babies. We saw her during the third pregnancy, and she was prediabetic. That meant a blood sugar

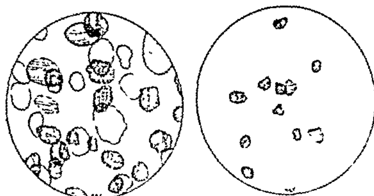


FIGURE 6. Surface islets of Langerhans. Reprinted, by permission, from Dubreuil, G. Hypertrophie du parenchyme endocrine du pancréas chez des nouveau-nés issus de mères glycosuriques. *Compt. rend. de l'Assoc. d'anat.* 46, 173 (1939).

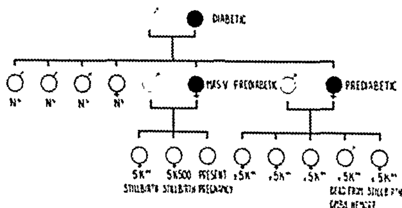


FIGURE 7. Mrs. S. 41 years 74K500. Reprinted by permission from Hoet, J. P. Carbohydrate metabolism during pregnancy. *Diabetes* 3, 1 (1954).

That patient is much more interesting when we consider her whole history, because she is the youngest surviving child of a person, still living, who has been diabetic from the age of fifty-five and who lost three babies through stillbirth. She does not know whether they were heavy or not, but probably the fact that they died so early means that they never could have become heavy newborns. She was the niece of two diabetics.

Thus, we see that the disease is very much involved, during pregnancy, with the death of some children, and the birth of children with pancreases which are abnormal. If the children survive, some are going to become diabetic. This points to a phenomenon which is well known by the clinicians, the anticipation of diabetes in children. The mother becomes diabetic after the child has been diabetic for many years.

Figure 9 demonstrates that we have to teach the obstetricians what is happening. Mrs. L. B. was a patient who had been seen by many obstetrical clinicians five or six years ago during her first pregnancy. This ended with toxemia. She was the niece of a diabetic woman. In December 1952, after eight weeks of pregnancy, a sugar tolerance test was made, which you see at 1. The blood sugar rises from 70 mg. to 120, going down after 120 minutes to 70 mg. To be on the safe side, plenty of dextrose was given to that patient. I saw her during the fifth month of pregnancy. At that time we see her blood sugar curve rising to 240 mg. in 90 minutes after a test dose of glucose. We then gave her from 50 to 60 units of insulin, which is a maximum dose, and she withstood it perfectly. The cesarean section was performed at the eighth month, and the birth was normal. As you see in curve 3, even during the patient's stay at the clinic she became normal again. I think that by giving lots of insulin during a prediabetic pregnancy, we protect the mother against a disturbance in her islets, and we hope that we also protect the newborn baby against hyperplasia of the islets of Langerhans.

Of course I know this interpolation to patients from experimental work is not a complete story. It contains many gaps, but it is of such importance to obtain the story of these patients that I took the liberty of introducing their histories into this conference on liver. I shall add that the animals to which we give 0.5 mg. of cortisone have lipemic blood six hours after the first injection, and that rats on the 16th or 17th day of pregnancy, if put on a fat diet, at once increase their excretion of acetone bodies from 15 mg. a day to 120 mg. a day, as Burn (23) has demonstrated.

above 200 mg. more than two hours after being given 50 gm of dextrose. She was diabetic during her pregnancy. She was given insulin, and the third baby is doing perfectly well It was born nearly two months ago We discovered also that her sister had the same condition. She also had had very heavy babies, and had lost two. The sister had a diabetic curve, and the mother of the two sisters had been diabetic as well

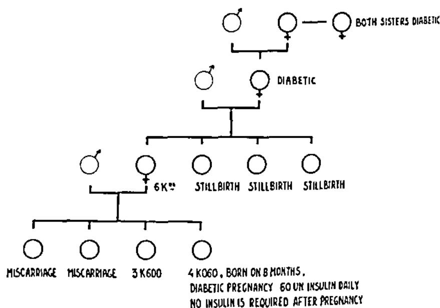


FIGURE 8 Mrs C Reprinted, by permission, from Hoet, J P Carbohydrate metabolism during pregnancy *Diabetes* 3, *1 (1954)*

Figure 8 shows another very typical history of Mrs C, who came to us because she had lost two babies There was a third child, born at eight months, and weighing 4 kg, which was living She herself weighed 6 kg at birth

We saw here during the fourth pregnancy She was prediabetic We gave her a large amount of insulin because the disturbance in carbohydrate metabolism is characterized by a very increased need for it Sixty units of insulin were injected daily during that last pregnancy

Shorr Protamine, or regular?

Hoet. In many cases we give protamine, but here, in this particular case, it was 30 units of regular insulin given twice daily However, very often we give protamine in one dose, with a small dose of regular at night

a rule, in a prediabetic rat, the pregnancy ends with the resorption of the fetus. They diminished the amount of alloxan given, but up to this moment have had very few newborn animals that have survived such a pregnancy. They have studied the offspring of alloxan pregnant diabetic rats, not only one pregnancy but a second and a third one, just as we observe humans, and they have a few animals living. Those animals have abnormal pancreases. That has been shown by Hultquist (25), in Gothenburg. However, Bartelheimer and Kloos observed, in the third and fourth months after birth, a hyperglycemia of 300 mg. in those animals which had had no contact whatever with alloxan. They got glycosuria, and they became what we call diabetic. They did not go as far as death, but rats are not the ideal species in which to study the progressive form of diabetes. However, I think this is a very important fact, because it shows that the maternal environment plays such a large role that the normal animal may become a diabetic animal which will have offspring with pathological pancreases. We may study

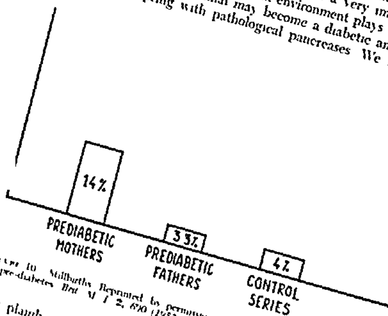


FIGURE 10. Stillbirths. Reprinted by permission, from Jackson, W. P. U.: Studies in pre-diabetes. Brit. M. J. 2, 690 (1952).

that plainly in the clinic (Figure 10), because we can compare the offspring of the diabetic father and mother. The offspring of the diabetic father never have still births, but when there is a diabetic mother there are stillbirths in 17 per cent of the cases (26).

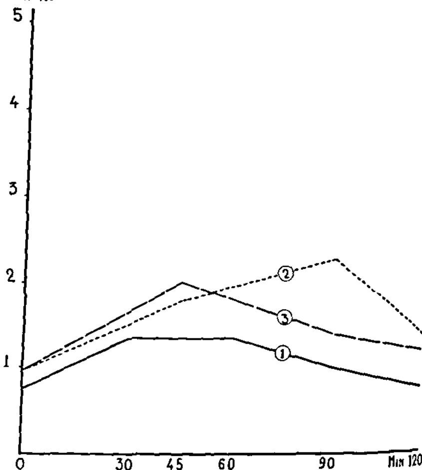


FIGURE 9 Mrs L B, 38 years Father's sister is diabetic Previous pregnancy has been toxemic (1) December 1952 2 months pregnant (2) March 1953 5 months pregnant (3) July 1953 1 month after delivery by cesarean section on 3rd June 1953, after 34 weeks of pregnancy.

Best I think it would be useful to say what happened in the alloxan diabetic rats

Hoet It might be stated as follows: the hypercorticalism of pregnancy, by way of the glycogen in the placenta, determines the nutritional economy of the fetus, which makes the embryonic pancreas abnormal This concept, derived from clinical studies, has been put to the test by two Germans, Bartelheimer and Kloos (24). As

Hoet. I think we may say that in the human it is really a reinforcement of the genetically touched pancreas. Bartelheimer and Kloos (21) demonstrated that when the pancreas is 100 per cent normal, and we alter the maternal environment of the mother, there will be offspring which become diabetic later even if there is no genetic factor coming in. Thus, what we observe in the clinic, by comparing diabetic mothers and fathers, is quite plain, there is an important role played by the mother who has been hyperglycemic during pregnancy.

Hanger: A woman on cortisone therapy would have to be very careful.

Hoet: Yes, but they may abort. In the hope of disturbing the formation of antibodies, cortisone was given to pregnant women in cases of rhesus incompatibility as a treatment, and quite a number aborted. The clinicians thought that it was the result of the rhesus incompatibility, but I suspect that it might have been due to the disturbance in carbohydrate metabolism. They did not check the blood sugar.

Best: Some observers might seize upon this as proof of the inheritance of acquired characteristics. The mother is diabetic, the offspring is diabetic. Obviously, however, it has nothing to do with that. It is the maternal environment that is important.

Shorr: Table III shows 25 children of diabetic fathers. If we multiply that by at least four, that would be 12 as compared with 25. There could be, as Dr Fremont-Smith suggested, a basic genetic factor responsible for 12, and the doubling of it could come from the environmental factor.

Hoet: This has been said by Dr Jackson (26), of Cape Town, South Africa, who is in Boston now. If we look at the figures shown in Figure 11, we immediately find the answer. They indicate that 17 per cent of the heavy newborns are attributed to the maternal environment, 9 per cent to the inheritance, and 5 per cent to unknown factors.

Shorr: Is there sometimes a combination of two factors?

Hoet: Yes. It is the reinforcement of the genetic lesion, as Dr Fremont-Smith puts it.

Shorr: This brings up a very important point. What criteria shall we use in the clinic to distinguish the prediabetic mother?

Hoet: I thank you for that question, Dr Shorr, because it is quite a clinical problem. It has been said that the first three months of pregnancy are characterized by renal glycosuria; therefore, my obstetrical colleagues say glycosuria has no pathologic significance.

TABLE III

105 Children of Diabetic Mothers Between 13 Months and 20 Years

Compared Growth of Boys and Girls		
Height	51 Boys	54 Girls
3 Inches Above Standard	57%	31%
Weight		
30 Lbs Above Standard	50%	20%
Glucose Tolerance Tests		
28 Are Hyperglycemic		
7 Are Hypoglycemic		
25 Children of Diabetic Fathers		
3 Are Hyperglycemic		
2 Are Hypoglycemic		

Reprinted, by permission, from White, P., Koshy, P., and Duckers, J The management of pregnancy complicating diabetes and of children of diabetic mothers M Clin North America 37, 1481 (1953), (Philadelphia and London, Saunders)

Table III shows the difference between the offspring of the diabetic father and the diabetic mother This was done by White, Koshy, and Duckers (27) I do not like to call them "diabetic mothers" when they were treated diabetic mothers; they were, rather, mothers with a nearly normal, but still pathologic, carbohydrate metabolism during pregnancy. We see that 28 of the 105 children had a hyperglycemic glucose tolerance test, and 7 had a definite hypoglycemic curve. Of the 25 children of diabetic fathers, only three had hyperglycemic curves, and only two had hypoglycemic curves.

Thus, there is an interplay between two factors. one, plain genetic heredity, and two, a very important factor, the maternal environment which we must recognize at an early stage and not wait until the woman is fifty and has permanent hyperglycemia. We must observe women at childbearing age and try to find out, long before they are permanently hyperglycemic, whether they have hyperglycemia of pregnancy, because this determines the condition of the child which later on will give an anticipation of diabetes.

Fremont-Smith. Would you say that in the child who has that genetic inheritance, and is also born of a diabetic mother, the two influences act together to produce a serious situation?

in Leiden. They were women who had lost at least one baby, who during pregnancy had prediabetic blood sugar curves, and to whom insulin in doses of 20, 40, and 100 units was given

Shorr: When did you start?

Hoet: At the fourth month. Now and then it was earlier, but at least by the fourth month. Thus, there is already quite strong clinical evidence that by changing the maternal environment, we protect the baby against stillbirth. We shall have to see what happens to children, but we hope that the phenomenon of anticipation of diabetes will be delayed

Shorr: In the next pregnancy you start from the very beginning?

Hoet: Yes.

Neefe. Are the miscarriages or abortions always late in the pregnancy?

Hoet: In plain prediabetic cases, it is late, the fetus dies *in utero* in the eighth month However, there are cases, on which I have clinical evidence, in which we must expect them to abort in the fourth month In one case, the first baby was born alive, the second, weighing eleven pounds, died two days after birth; and the third, fourth and fifth pregnancies were abortions at the fourth month Of course, we have all types of cases.

Best: Dr Houssay, have you studied pregnancy in partially depancreatized animals?

Houssay: With proper support, an animal subjected to pancreatectomy can be maintained in a prediabetic state When pregnant, such an animal has, in the beginning, a normal blood sugar level The blood sugar tolerance curve is normal, but after a short time, the curve will go higher and higher Then the animal develops glycosuria

Thus, the first sign of diabetes in this animal is in the tolerance curve, which is the only possible means of detection We have some observations but we have not yet finished In alloxan diabetes, there are sometimes lesions in the placenta in cases of stillbirth However, we have not yet seen the increase in weight in the newborn and in rats, we do not yet know the reasons for these negative results

Hoet The only people who have seen it in rats are Hultquist and Engfeldt (25,28) in Gothenburg They have seen a few cases of a distinct increase in weight in rats fetuses who died *in utero* the last day, however, as a rule, the increase in weight cannot be observed because they die off during pregnancy There was one rat, the photograph of which is well known, which weighed 8 gm, whereas the usual weight is from 5 to 6 gm On the other hand, in the

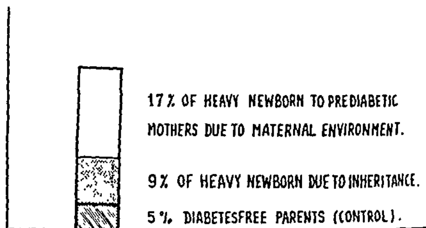


FIGURE 11 Role of maternal environment on overweight babies. Reprinted, b permission, from Jackson, W. P. U.: Studies in pre-diabetes *Brit. M. J* 2, 6 (1952)

during pregnancy. When they see a heavy newborn, they contr a sugar tolerance curve during post partum, which is one of th worst criteria one could think of. During post partum the woma has a physiological hypoglycemia. Fasting, she has a blood sug content of between 60 and 80 mg. If one gives 50 gm. of dextros the first ten days after delivery, the blood sugar does not go highe than 125 mg., and comes back to 60 or 70 mg. after 90 minutes Therefore, the obstetricians who obtain a blood sugar curve during the ten days that the post partum woman is in the lying-in hospital, do so at the very moment when they cannot discover the diabetes The first three months are also not the months with hypercorticalism As has been shown by Venning and Browne (15) and others, hypercorticalism starts from the fourth month onwards, comes to a peak at the sixth month, is a bit lower at the seventh, starts to increase again at the eighth month, and then the last week before delivery goes down again. Thus, the criterion for prediabetes is the sugar tolerance curve done from the fourth month of pregnancy It is best to do it in both the second and third trimesters of pregnancy.

Shorr. In other words, damage to the fetus is not done in the first four months?

Hoet I could give you the figures from twenty personal cases, and also three cases from A. J. C Haex* in the clinic of Mulder

* Personal communication

in Leiden. They were women who had lost at least one baby, who during pregnancy had prediabetic blood sugar curves, and to whom insulin in doses of 20, 40, and 100 units was given.

Shorr: When did you start?

Hoet: At the fourth month. Now and then it was earlier, but at least by the fourth month. Thus, there is already quite strong clinical evidence that by changing the maternal environment, we protect the baby against stillbirth We shall have to see what happens to children, but we hope that the phenomenon of anticipation of diabetes will be delayed

Shorr: In the next pregnancy you start from the very beginning?

Hoet: Yes

Neefe: Are the miscarriages or abortions always late in the pregnancy?

Hoet: In plain prediabetic cases, it is late; the fetus dies *in utero* in the eighth month However, there are cases, on which I have clinical evidence, in which we must expect them to abort in the fourth month In one case, the first baby was born alive, the second, weighing eleven pounds, died two days after birth, and the third, fourth and fifth pregnancies were abortions at the fourth month. Of course, we have all types of cases

Best: Dr Houssay, have you studied pregnancy in partially depancreatized animals?

Houssay. With proper support, an animal subjected to pancreatectomy can be maintained in a prediabetic state When pregnant, such an animal has, in the beginning, a normal blood sugar level The blood sugar tolerance curve is normal, but after a short time the curve will go higher and higher. Then the animal develops glycosuria

Thus, the first sign of diabetes in this animal is in the tolerance curve, which is the only possible means of detection We have some observations but we have not yet finished In alloxan diabetes, there are sometimes lesions in the placenta in cases of stillbirth However, we have not yet seen the increase in weight in the newborn and in rats we do not yet know the reasons for these negative results

Hoet The only people who have seen it in rats are Hultquist and Engfeldt (2528) in Gothenburg They have seen a few cases of a distinct increase in weight in rats fetuses who died *in utero* the last day however, as a rule, the increase in weight cannot be observed because they die off during pregnancy There was one rat, the photograph of which is well known, which weighed 8 gm., whereas the usual weight is from 5 to 6 gm On the other hand, in the

adrenalectomized animals Dr. Houssay (29) observed, in 1945, that the weight of the animals was low for one or two pregnancies, and that it increased again when the accessory adrenals took up the function of the adrenals.

Houssay: In these cases, however, when the rats are born their weight is slightly under normal, and during lactation the growth is deficient. The weight of the adrenals is increased in the newborn It has been definitely established that adrenotropin increases after adrenalectomy.

Hoet: We have done another experiment, not on the absolute weight, but on the increase in weight after four days of cortisone, and there we obtained an increase in weight of the fetus. When we gave 0.5 mg of cortisone from the twentieth day of pregnancy to the twenty-fourth, there was not a gain of glycogen in the placenta; it was down to 1 gm. or under. However, the weight of the fetus increased more quickly It is difficult to compare two different animals, and the absolute weights of the fetuses

Houssay: Also, if the birth is delayed, in some instances the fetuses die. However, if they are born, they are larger because the pregnancy has been extended from one to three days.

Hoet. Yes, that is one of the difficulties Therefore, we gave 0 5 mg of cortisone for only four days We obtained an increase, but the figures are not absolutely certain The mean weight of the fetuses was 2 12 gm for twelve fetuses in the control, and 2 70 gm for twelve fetuses in the experimental animal.

Shorr: To what extent is this increase in weight in water?

Hoet. I could not tell you.

Best What is the sequence of events here? Pregnancy is a stress?

Hoet: Increase in corticoids.

Best: Yes, but what starts the increase in corticoids?

Houssay Could it be estrogens?

Hoet Estrogens or anterior pituitary hormones.

Popper Formation of hormones in the placenta itself may be important.

Hoet It is obtained with ACTH

Kosterlitz Can you produce the diabetogenic effect of pregnancy by removing the fetuses and leaving the placentae?

Hoet. I have not done that experiment

Kosterlitz This may tie up with observations on the liver, because in pregnancy of rodents (mice and rats) we find that from the second half of pregnancy onward, there is a very marked change in the liver There is increased cytoplasmic basophilia, which is

due to an increase in the ribonucleic acid content; this occurs in the absence of the fetuses as long as the placentae are intact. There seems to be a factor secreted by the placentae and I was just wondering whether your phenomenon is of a similar nature.

Other changes one finds in the liver, such as an increased turnover of phospholipid P, are dependent upon the presence of the fetuses When they are removed, leaving the placentae intact, this increased turnover is abolished Thus, we have in pregnant organisms various changes, some dependent upon the internal secretions of the placentae, and others on the increased metabolism due to the presence of the fetuses It would be very interesting to know into which category your observation fits.

Hoet: I think that experiment will be very important, because there are very good reasons to think that the placenta may be the source of ACTH I do not say that Opsahl and Long's (30) figures are absolute, but they have been showing that the placenta itself contains, and probably produces, a large amount of ACTH.

Shorr. Jailer (31) has evidence on that

Hoet: Yes, and also Tarantino (32) and Gray (33) However, there are at least three laboratories which have shown that the placenta is a source of ACTH Then we have what you described. even though the fetus is removed, if the placenta goes on living the source of ACTH is there Then we have the phenomenon of the hyperglycemia if the pancreas is not perfect

Kosterlitz We have done some experiments in that direction We did not find any evidence of ACTH, but perhaps our evidence was inconclusive When we hypophysectomize pregnant rats and remove the fetuses at the same time, then the weight of the adrenals is reduced just as it is in nonpregnant animals. Thus, the weight of the adrenals is not maintained by a secretion of the placenta, which would indicate

Hoet But you remove the fetuses?

Kosterlitz Yes, but the placentae are still there

Hoet Is that done during the last week of pregnancy?

Kosterlitz Yes

Hoet Then the fetuses are formed The figures which I reported concern the stage in which the placentae are large and the fetuses small If the placenta does make ACTH, it would not be during the last week when the organs of the fetus are there, it would be during the beginning and middle of pregnancy

Kosterlitz We removed the fetuses on the thirteenth or fourteenth day of pregnancy in the rat They are very small at that

stage We were not able to maintain the weight of the adrenals

Hoet. We do not fully know the physiological mechanisms, but the high susceptibility of the pregnant animal to carbohydrate metabolism disturbance produced by glucocorticoids is quite an astonishing fact. Going into that, we found that Florentin and Picard (34), and Rosenloecher (35) described the formation of new islets in the pancreas during gestation Verne (36), from Paris, is able to distinguish a gestation pancreas from any other pancreas We think that pancreases of pregnant animals are macroscopically a little more reddish than normal

Shorr: I wonder whether the sensitivity might be related to the concurrent action of the steroids For example, during the luteal phase, with high progesterone production, the glycogen content of the uterine cells is greatly increased, and if there are two factors operating, one of which is a hormonal setting already making for glycogen deposition, then we have a situation of increased sensitivity to any factor that will deposit glycogen. We cannot dissociate these, naturally, in a pregnant animal.

Hoet: It is possible However, there is a very close functional relationship between the islets of Langerhans and the secretion of glucocorticoids by the adrenals. Green, *et al.* (37) have found that in injecting more and more glucocorticoids, they also have to increase in a parallel way the dosage of insulin.

Gyorgy: Can a gestation pancreas be produced in a nonpregnant animal with glucocorticoids?

Hoet: I suppose it can. Haist (38), who injected cortisone and measured the number of islets, found quite an increase after injecting cortisone for a few days.

Hoffbauer. Was an actual increase in the total number of islets in the pancreas observed?

Hoet: Yes, but I think the phenomenon is even more striking than Haist thinks The islets are really very hyperplastic; Kobernick and More (39) have shown that they are very rich in glycogen

Hoffbauer: Then it is hyperplasia of islets that already exist, not newly formed islets?

Hoet: I could not tell you that

Lillie: Is it hyperplasia or an enlargement?

Hoet. There is a very clear-cut enlargement, but there is also hyperplasia.

Best: What is the story? The placenta forms Either it makes ACTH or stimulates the liberation from the pituitary. We obtain

an increased secretion of glucocorticoids, which stimulates the pancreas You go on from there, Professor Hoet

Hoet: If the pancreas is normal, it keeps the blood sugar tolerance absolutely normal, and there is a normal pregnancy. If it is an animal that has received alloxan experimentally, a young person in a family in whom the pancreas is genetically a bit less functional, that pancreas is not able to adapt itself. Therefore, the maternal environment becomes hyperglycemic, and the glycogen is not formed in the right way in the placenta. Probably there is a hyperglycemia during the embryogenesis of the pancreas That pancreas becomes abnormal macroscopically, we can see it. If the newborn lives, that pancreas, which has been hyperplastic during its embryogenesis, is going to become an organ which gives way at the age of fifteen, twenty-five, or later, when infection or some other factor occurs Thus, I think we have a link between the mother and the anticipation of diabetes in children, which is not genetic, but a maternal environmental factor that may be corrected

In the book of S. Warren (40), there is a picture of the islets of Langerhans full of glycogen, produced by the injection of an amount of cortisone which was not very large, but still much larger than what we have been using

Goldblatt Probably erroneously, I was under the impression that, as in the case of the glomerulus, so with the islets of Langerhans, the number actually does not increase after birth. I have thought of the enlargement as being due to an increase, either in the size of individual cells, or in the number of cells

Best This is before birth

Hoet Also in the mother's pancreas Verne (36) showed pancreases in which there were clearcut increases in the number of islets

Goldblatt I am fully aware that the number of islets in different individuals varies a great deal, that is unquestionably correct I am also willing to accept the hypothesis that there is a change from a small number to a large number, but it is new to me.

Lillie I think it would be hard to prove.

Hoet By using pregnant and nonpregnant mice of the same strain it has been shown that in pregnancy there is an increased number of islets

Popper I should like to raise several points The first deals with the pancreas, and may offer an answer to the question which Dr Goldblatt has raised. If new islets are formed, as I believe doubtlessly happens under experimental conditions and may also occur

stage. We were not able to maintain the weight of the adrenals

Hoet: We do not fully know the physiological mechanisms, but the high susceptibility of the pregnant animal to carbohydrate metabolism disturbance produced by glucocorticoids is quite an astonishing fact. Going into that, we found that Florentin and Picard (34), and Rosenloecher (35) described the formation of new islets in the pancreas during gestation. Verne (36), from Paris, is able to distinguish a gestation pancreas from any other pancreas We think that pancreases of pregnant animals are macroscopically a little more reddish than normal.

Shorr: I wonder whether the sensitivity might be related to the concurrent action of the steroids. For example, during the luteal phase, with high progesterone production, the glycogen content of the uterine cells is greatly increased, and if there are two factors operating, one of which is a hormonal setting already making for glycogen deposition, then we have a situation of increased sensitivity to any factor that will deposit glycogen We cannot dissociate these, naturally, in a pregnant animal.

Hoet. It is possible. However, there is a very close functional relationship between the islets of Langerhans and the secretion of glucocorticoids by the adrenals Green, *et al.* (37) have found that in injecting more and more glucocorticoids, they also have to increase in a parallel way the dosage of insulin.

Gyorgy· Can a gestation pancreas be produced in a nonpregnant animal with glucocorticoids?

Hoet: I suppose it can. Haist (38), who injected cortisone and measured the number of islets, found quite an increase after injecting cortisone for a few days.

Hoffbauer. Was an actual increase in the total number of islets in the pancreas observed?

Hoet. Yes, but I think the phenomenon is even more striking than Haist thinks. The islets are really very hyperplastic; Kobernick and More (39) have shown that they are very rich in glycogen

Hoffbauer. Then it is hyperplasia of islets that already exist, not newly formed islets?

Hoet I could not tell you that.

Lillie Is it hyperplasia or an enlargement?

Hoet There is a very clear-cut enlargement, but there is also hyperplasia

Best What is the story? The placenta forms. Either it makes ACTH or stimulates the liberation from the pituitary We obtain

an increased secretion of glucocorticoids, which stimulates the pancreas You go on from there, Professor Hoet

Hoet. If the pancreas is normal, it keeps the blood sugar tolerance absolutely normal, and there is a normal pregnancy. If it is an animal that has received alloxan experimentally, a young person in a family in whom the pancreas is genetically a bit less functional, that pancreas is not able to adapt itself. Therefore, the maternal environment becomes hyperglycemic, and the glycogen is not formed in the right way in the placenta Probably there is a hyperglycemia during the embryogenesis of the pancreas That pancreas becomes abnormal macroscopically, we can see it If the newborn lives, that pancreas, which has been hyperplastic during

and the anticipation of diabetes in children, which is not genetic, but a maternal environmental factor that may be corrected.

In the book of S Warren (40), there is a picture of the islets of Langerhans full of glycogen, produced by the injection of an amount of cortisone which was not very large, but still much larger than what we have been using

Goldblatt. Probably erroneously, I was under the impression that, as in the case of the glomerulus, so with the islets of Langerhans, the number actually does not increase after birth I have thought of the enlargement as being due to an increase, either in the size of individual cells, or in the number of cells

Best This is before birth

Hoet Also in the mother's pancreas. Verne (36) showed pancreases in which there were clearcut increases in the number of islets

Goldblatt I am fully aware that the number of islets in different individuals varies a great deal, that is unquestionably correct I am also willing to accept the hypothesis that there is a change from a small number to a large number, but it is new to me

Lillie I think it would be hard to prove

Hoet By using pregnant and nonpregnant mice of the same strain, it has been shown that in pregnancy there is an increased number of islets

Popper I should like to raise several points The first deals with the pancreas, and may offer an answer to the question which Dr. Goldblatt has raised If new islets are formed, as I believe doubtlessly happens under experimental conditions and may also occur

stage. We were not able to maintain the weight of the adren

Hoet: We do not fully know the physiological mechanisms the high susceptibility of the pregnant animal to carbohydr metabolism disturbance produced by glucocorticoids is quite astonishing fact. Going into that, we found that Florentin a Picard (34), and Rosenloecher (35) described the formation new islets in the pancreas during gestation Verne (36), from Par is able to distinguish a gestation pancreas from any other pancrea We think that pancreases of pregnant animals are macroscopicall a little more reddish than normal.

Shorr: I wonder whether the sensitivity might be related to th concurrent action of the steroids For example, during the lutea phase, with high progesterone production, the glycogen content o the uterine cells is greatly increased, and if there are two factors operating, one of which is a hormonal setting already making for glycogen deposition, then we have a situation of increased sensitivity to any factor that will deposit glycogen We cannot dissociate these, naturally, in a pregnant animal.

Hoet: It is possible. However, there is a very close functional relationship between the islets of Langerhans and the secretion of glucocorticoids by the adrenals Green, *et al* (37) have found that in injecting more and more glucocorticoids, they also have to increase in a parallel way the dosage of insulin.

Gyorgy Can a gestation pancreas be produced in a nonpregnant animal with glucocorticoids?

Hoet. I suppose it can Haist (38), who injected cortisone and measured the number of islets, found quite an increase after injecting cortisone for a few days.

Hoffbauer Was an actual increase in the total number of islets in the pancreas observed?

Hoet. Yes, but I think the phenomenon is even more striking than Haist thinks The islets are really very hyperplastic; Kobernick and More (39) have shown that they are very rich in glycogen

Hoffbauer. Then it is hyperplasia of islets that already exist, not newly formed islets?

Hoet I could not tell you that

Lillie Is it hyperplasia or an enlargement?

Hoet There is a very clear-cut enlargement, but there is also hyperplasia

Best What is the story? The placenta forms. Either it makes ACTH or stimulates the liberation from the pituitary. We obtain

you that Emmanuel C Ambrose,* of the University of London, told me that the best material for study was the pregnant mare, in which the islets increased many times

The fact of erythroblastosis is a clinical one They have nucleated red blood cells at the periphery, perhaps 20 per cent. I had one with 10 per cent If they died, the pathologists described erythroblastosis in the liver, which means islets of blood-forming material The most complete paper on this subject is, I think, by Miller (21) We saw the history of the two patients, whose two children had died The two newborns who died had been exsanguinated and transfused, because the medical men thought it was rhesus incompatibility, it was at the next pregnancy that we were able to put right that false diagnosis

I do not think we can say that experimentally we have seen erythroblastosis, up to now it has been only a clinical fact However, I know that Gyorgy has been looking into the matter, and has some speculations about the pathogenesis of that condition

Gyorgy As Professor Hoet stated, Dr H C Miller (42) of Kansas City, about nine years ago also published an excellent paper on erythroblastosis in newborn infants of diabetic and prediabetic mothers, with all the histologic criteria one would require I cannot remem[illegible] placenta, but certainly [illegible] In his conclusions he sta[illegible]n a newborn of a diabetic or prediabetic mother, cannot be distinguished from the similar condition seen in rhesus incompatibility In the latter, as Professor Hoet pointed out, the important difference is that the erythroblastosis and its sequelae really come to the fore on the third or fourth day, sometimes on the second day, but less commonly at birth, whereas in diabetic erythroblastosis, it disappears in a few days and does not require any treatment.

Erythroblastosis means increased destruction of red blood cells, which in turn indicates exposure to some hematotoxic agent We found previously that alloxan, or rather its reduction product, dialuric acid, may act as a hemolyzing agent provided there exists, simultaneously, a deficiency of vitamin E (43,44,45) Furthermore, it has been shown that newborn animals and humans are in a state of more or less pronounced vitamin E deficiency, which may be called "physiologic" (43,46) In order to connect these two seemingly incongruous findings, we made the assumption that in diabetic or prediabetic mothers there must circulate a hemolyzing, alloxan-

* Personal communication 1953

exceptionally in the human, the intercalated cells of the excretory parenchyma should be the site of the formation of new islets For instance, these cells proliferate in cystic fibrosis of the pancreas, giving rise to the formation of islet-like structures, as I am sure Dr. Goldblatt and Dr. Lillie will agree. If new islets should form in pregnancy, these intercalated cells should carry the brunt of regeneration and new formation, and I believe it should not be difficult for the morphologist to demonstrate such an alteration

The second point deals with the very fascinating relationship between the placenta and liver. My co-worker, Dr. Elias, has recently restudied the embryology of the liver, and I have learned from him that the liver eventually takes the place of the yolk sac With an apology to the physiologists, I believe with him that the liver has a function probably comparable to that of the yolk sac even in adult life. The metabolic interrelationships between liver and placenta should therefore be very interesting.

The third point deals with the erythroblastotic picture in the diabetic child, which I still do not quite understand, I should appreciate enlightenment from Professor Hoet or Dr. Gyorgy. How do we define erythroblastosis? Of course, the common definition is that of a hemolytic disease of the newborn due to incompatibility, mostly in the Rh group, but sometimes in other blood group factors, the fetal and infantile organism responding with excessive erythropoietic regeneration. Is there evidence of hemolysis or any other reason for such a regeneration? Morphologically, we have, I believe, three important criteria for erythroblastosis There is the picture of excessive erythropoiesis in the liver, which of course occurs in other conditions also and I believe is that to which you refer. Next, the septal capillaries of the lung reveal in erythroblastosis very large erythropoietic cells, and this is almost more characteristic than the changes in the liver, and even recognized in macerated fetus We often rely more on the lungs than the liver for diagnosis, and I wonder whether these changes are seen in diabetes

Lastly, the placenta may show a characteristic picture of thickened villi, though it is claimed that that occurs mainly in edematous children (41) Is the glycogen increased in the placenta in erythroblastosis? I am confused about the relation between diabetes or the glucocorticoid response and the erythroblastotic changes in the liver Could you help me, Professor Hoet?

Hoet Before speculating any more, we hope to have many more morphological data, we do not know exactly the source of the new islets, and so on, and are looking for material. However, I can tell

a girl who became pregnant. In the third month she had a blood sugar of 300 mg She aborted. When she came back, she was plainly diabetic, and she remained so indefinitely. Apparently, the hypoglycemic curve may change to a hyperglycemic curve, and then back again to a hypoglycemic curve

Fanconi (1935-1938), published reports on six cases of acetonemia with hypoglycemia, and he published these because after the phase of hypoglycemia they became plainly diabetic. I, myself, have seen two cases of chronic hypoglycemia not in the convulsive stage, they were the children of diabetic mothers One was born when the mother died of acidotic coma during pregnancy

That is not a proof We have no pathological material However, when we see those patients with hypoglycemia for years and years, and they become diabetic later, it is a fact which we cannot forget, even if we do not know how it happens.

Hartroft. What do you think happens to all these superabundant islets in later life when diabetes develops?

Hoet I do not know

Shorr: Inasmuch as diabetes is not simply an insulin insufficiency, [illegible]

Dr Houssay?

Houssay That is possible, it seems to me that the point deserves more quantitative research

Best Is there any clinical evidence that the hypercorticalism persists? That would be in the mother, would it not?

Houssay With cortical hormones, after a long time we obtain a hyperplasia of the islets in the rat We could prevent diabetes in rats with subtotal pancreatectomy by giving different compounds At the beginning there is an increase of symptoms of diabetes in some cases because the cortical steroids have some diabetogenic action, but after three, four, five, or six months, the animals treated have less diabetes than the untreated For example, they have 10 per cent, or something like that, and the animals without treatment have 77 per cent In the protected rats, there is an increase in the islets of Langerhans I have data on compounds A, E and F

Madden. I believe there are some figures on the number of islets in the pancreas of the newborn in relation to the adult human pancreas, and these might be pertinent. As I recall, they are quoted by George Gomori (48), but I do not remember whom he was quoting After birth there [illegible] distinct increase in the number of

like agent, with its source in the maternal metabolism. The agent may freely invade the circulation of the fetus, where its hemolyzing effect will become evident owing to the absence of sufficient amount of protective vitamin E. On the other hand, the red blood cells of the mother, with her sufficient stock of vitamin E, will and should remain unharmed. The disappearance of erythroblastosis in the infant after birth may be explained by cutting off the noxious agent in its source, i.e., in the maternal metabolism.

Obviously there is no proof for this assumption. Giving large doses of vitamin E to pregnant mothers failed in experiments because of the inability to break through the placental barrier for vitamin E. In contrast to rats, the "physiological" vitamin E deficiency in newborn human infants was not significantly relieved by administration of large doses of vitamin E to the pregnant mother. We should perhaps try to substitute some other antioxidant for vitamin E, a substance which might penetrate the placental barrier, and act in the fetus as vitamin E does.

Hanger: Do these prediabetic children have evidence of hemolysis?

György: No, because it is not a massive hemolysis Jaundice is rare, and usually not very marked in the erythroblastosis of newborn infants of diabetic or prediabetic mothers. As a rule the only clinical sign of erythroblastosis is the presence of nucleated red blood cells in pathologically large numbers in the circulating blood, with extramedullary hematopoiesis at histological examination of the liver and lungs

Hartroft: The question I should like to ask Professor Hoet is one that he may say is premature at this stage, but nevertheless I hope he will consider it He demonstrated that the heavy newborn of the prediabetic mother has more islets than normal, as shown by the figures, and said that this is associated with hypoglycemia Later on in life, some of these infants develop hyperglycemia and diabetes

This last step I find difficult to comprehend. If an infant is born with even better than normal equipment for the production of insulin, why should its islets fail later on? This sequence seems at variance with the classical concepts of disuse atrophy. I wonder whether Professor Hoet could explain the link between these two stages of hyperplasia and atrophy.

Hoet: There is a link in the experience of certain clinics Dr John (47) who, from his experience of 15 to 20 years published a paper on 57 cases of prediabetics in 1950, has shown seven or eight cases who were hypoglycemic for five or six years. One of them was

and were plainly hypoglycemic Then they became pregnant. They were all right during pregnancy, but after partus, the convulsions again started in both cases. Both the cases of Campbell, and the one of Pompen, were operated upon and very clear-cut adenomata, or insulinoma were found

When we look at the blood sugars during pregnancy, or at incidences of infection, and so on, we find that even during the convulsive period of hypoglycemia, there are hyperglycemic tolerance curves Thus, hypoglycemia and hyperglycemia are conditions which have something in common. "Dysinsulinism" has been used very often to cover all variances in the relations of our clinical statements to the pathological findings

Herman Ferner (52) in 1938, saw that 40 per cent of the cells of islets in normal newborn children are beta cells, and in the pancreas of the newborn of diabetic mothers, the figure is still increased I think his statements are fairly well established Dr. Hartroft told me that Ferner used drawings and not microphotographs, but I still think it was worth while to show them in his book

Neefe Is there any possibility that in the hypoglycemic states that you referred to one might be dealing with an aberrance of external pancreatic secretion in these newborns, rather than just the islets of Langerhans? For instance, in deficiencies of extrapancreatic secretions, at times one does see hypoglycemia as a phenomenon It may be that the total organ is involved in these figures

Hoet Of course, these are statements out of the literature However, if we read the literature on hypoglycemia and hyperglycemia, we find the clinical fact that cancer of the head of the pancreas produces diabetes One gives these cases insulin for four or five months, but the last two months they do not wish any insulin, and become plainly hypoglycemic Verne, and others, have been studying those cases, which are more or less a repetition of the ligation of the pancreatic duct by Laguesse (53) and others, and they find an increase in islets in the pancreas, when the head is cancerous

Of course, we should not try to go too far, and this brings us a long way from the statement that during pregnancy the woman is hypersensitive, from the carbohydrate metabolism point of view, to cortisone and ACTH

REFERENCES

1 MAJOR, S G, and MANN, F C Formation of glycogen following pancreatectomy *Am J Physiol* 102, 409 (1932)

islets. If this reserve, so to speak, were used up by the stimulus occurring in a fetus of a diabetic mother, it might be ready for the assaults of later life

Hartroft: You think the pancreas has only a certain reserve for forming islets, if it is exhausted too soon, there is nothing left.

Madden: There are certain variable limitations for reserves of all the organs; for example, hypertrophy of the heart. The heart does not hypertrophy indefinitely; there are limitations What imposes them, I certainly do not know. This just occurs as a possibility. If the hyperinsulinism occurs early in life, it may, as Dr Shorr suggested, induce a Frankenstein reaction of the anti-insulin balance that may have a greater capacity and could, therefore, easily dominate later on, going from a hyperinsulin and hypoglycemic state, to a hyperglycemic state.

Lillie: Is there anything known about any shift in cell type of the islets in the alpha and beta cells?

Madden: I have not seen that

Houssay: Usually in the hyperplasia there are more beta than alpha cells in these cases

Goldblatt: There is one assumption that we are all making which perhaps may not be justifiable; that is, that with the increase in number, the cells are still normal In that case it may be that the cells are actually more vulnerable to noxious influences, and therefore deteriorate more readily

Houssay: An important question is the animal species studied The rat very easily develops a hypertrophy and an increase of the islets. However, observations on the rat do not necessarily indicate what could happen in other species.

Best If we can reproduce this in an experimental animal, it should be reasonably easy to work out some of the mechanisms

Hoet. As to the relationship between chronic hypoglycemic states and diabetes, I should like to report two very important observations One is the first case of hypoglycemia was operated on by Judd (49), of the Mayo Clinic. The patient was using 200 gm of sugar a day, and was operated upon for his chronic convulsive hypoglycemia. They found a tumor in the islets of Langerhans, but he died after the operation Three years before he was in convulsive hypoglycemia, he was passing sugar in the urine.

There are two cases in the literature, one published by Pompen (50), from Deventer, and another by Howland, Campbell and collaborators (51), of hypoglycemic women who started convulsions the first fortnight after partus. They had a blood sugar of 0 3

to the chemistry of growth *Proc Roy Soc, London*, B 80, 263 (1908)

19 HOET, P L Cortisone and placental glycogen in the rabbit *J Physiol* 120, 68P (1953)

20 VAN BEEK, C Autopsy findings in stillbirths and neonatal deaths suggesting maternal diabetes *Communication 1st Internat Congr of the I D F, Leiden, 1952.*

21 MILLER, H C, and WILSON, H M: Macrosomia, cardiac hypertrophy, erythroblastosis and hyperplasia of the islands of Langerhans in infants born to diabetic mothers *J Pediat.* 23, 251 (1943)

22 DUBREUIL, G Hypertrophie du parenchyme endocrine du pancréas chez des nouveau nés issus de mères glycosuriques *Compt rend de l'Assoc d anat* 46, 173 (1938)

23 BURN, J H *The Background of Therapeutics* New York and London, Oxford, 1948

24 BARTELHEIMER, H, and KLOOS, K Die Auswirkung des experimentellen Diabetes auf Gravidität und Nachkommenschaft *Ztschr f d ges exper Med* 119, 246 (1952)

25 HULTQUIST, G T Diabetes and pregnancy, animal study *Acta path et microbiol scandinav* 27, 695 (1950)

26 JACKSON, W P U Studies in pre diabetes *Brit M J* 2, 690 (1952)

27 WHITE, P, KOSHY, P, and DUCKERS, J The management of pregnancy complicating diabetes and of children of diabetic mothers *M Clin North America* 37, 1481 (1953) (Phila delphia and London, Saunders)

28 HULTQUIST, G T, and ENGFELDT, B Giant growth of rat fetuses produced experimentally by means of administration of hormones to the mother during pregnancy *Acta endocrinol* 3, 365 (1949)

29 HOUSSAY, B A Acción de la insuficiencia suprarrenal durante la preñez sobre la madre y el hijo *Rev Soc argent de biol* 21, 316 (1945)

30 OPSAHL, J C, and LONG, C N H Identification of ACTH in human placental tissue *Yale J Biol & Med* 24, 199 (1951)

31 JAILER, J W *Adrenal Function During Pregnancy and the Effect of ACTH During Pregnancy* Proc Second Clin ACTH Conf Vol I Research New York, Blakiston, 1951 (p 77)

32 TARANTINO, C Sulla presenza di ACTH nella placenta *Folia endocrinol* 4, 197 (1951)

33 DE COURCY, C, GRAY, C H, and LUNNON, J B Adrenal cortical hormones in human placenta *Nature* 170, 494 (1952)

34 FLORENTIN, P, and PICARD, D Recherches sur le pancreas endocrine *Rev franç d endocrinol* 14, 1 (1936).

35 ROSENLOECHER, K Die Veranderungen des Pankreas in der Schwangerschaft bei Mensch und Tier *Arch f. Gynak.* 151, 567 (1932)

36 VERNE, J · Polynesie et macronésie langerhansiennes; considérations histophysiologiques sur l'augmentation de nombre et de

2. BARKER, S B, and SWEET, J E: Effects of carbohydrate plethora in experimental diabetes. *Science* 86, 270 (1937).
3. KOSTERLITZ, H.. Über die Glykogenbildung in der Leber ohne Insulin; zugleich ein Beitrag zur Theorie der Ersatzkohlehydrate *Arch f. exper. Path. u. Pharmakol* 173, 159 (1933).
4 KRAHL, M E.· Effect of insulin and pituitary hormones on glucose uptake in muscle. *Ann. New York Acad. Sc.* 54, 649 (1951)
5. SHORR, E.: Restoration of carbohydrate oxidation in diabetic tissue in vitro *Science* 85, 456 (1937).
6. DE DUVE, C, HERS, H G., and BOUCKAERT, J. P.: Nouvelles recherches concernant l'action de l'insuline; action de l'insuline sur le glycogène du foie. *Arch internat. pharmacodyn.* 72, 45 (1946).
7 DE DUVE, C, DE NAYER, P. P., VAN OOSTVELDT, M, and BOUCKAERT, J P.: Nouvelles recherches concernant l'action de l'insuline, action de l'insuline chez l'animal normal, hépatectomisé et éviscéré *Arch. internat. pharmacodyn.* 70, 78 (1945)
8 HIMWICH, H E. *Brain Metabolism and Cerebral Disorders* Baltimore, Williams & Wilkins, 1951
9 KRUHØFFER, P., and MUNTZ, J. A · Carbohydrate metabolism of the isolated perfused cat liver as studied by labelled glucose and fructose *Acta physiol. scandinav.* 30, 258 (1954)
10 STETTEN, DEW, JR, and BOXER, G E: Studies in carbohydrate metabolism, metabolic defects in alloxan diabetes *J Biol Chem.* 156, 271 (1944).
11 SOSKIN, S, ESSEX, H. E, HERRICK, J F., and MANN, S C. Mechanism of regulation of blood sugar by liver *Am J Physiol* 124, 558 (1938)
12 LOEWI, O Die chemische Uebertragung der Nervwirkung *Schweiz med Wchnschr* 67, 850 (1937).
13 BARNS H. H F, LINDAN, O, MORGANS, M E., REID, E, and SWYER, G I M Foetal mortality in pregnant rats treated with anterior-pituitary extracts and in alloxan-diabetic rats *Lancet* 2, 841 (1950)
14 YOUNG, F G Growth hormone and experimental diabetes *J. Clin Endocrinol* 11, 531 (1951)
15 VENNING, E H, and BROWNE, J S L.: Urinary excretion of adrenal cortical steroids *Ann New York Acad Sc.* 50, 627 (1949)
16 NELSON, D H, SAMUELS, L T, WILLARDSON, D. G, and TYLER, F H The levels of 17-hydroxycorticosteroids in peripheral blood of human subjects *J Clin Endocrinol* 11, 1021 (1951)
17 BUSH, I E The paper chromatography of steroids and its application to assay problems *Bioassay of Anterior Pituitary and Adrenocortical Hormones* Ciba Foundation Colloquia on Endocrinology Vol V London, Churchill, 1953 (p 203)
18 LOCHHEAD, J, and CRAMER, W. The glycogenic changes in the placenta and the foetus of the pregnant rabbit· a contribution

THE LIVER AND FAT METABOLISM

SAMUEL GURIN
Department of Physiological Chemistry
University of Pennsylvania School of Medicine

It is quite apparent that the problems of the liver and fat metabolism cannot be confined to anabolism and catabolism of fat. Fat mobilization, transport from extrahepatic tissues to the liver, and hormonal and physiological controls are also involved. There is the question of release of lipid from the liver to the circulation. There are other problems, involving the effects of nutritional deficiencies upon the fat content of the liver. All of these, of course are tremendously important.

Can any of these be pushed along another angstrom or so by utilizing some of the newer tools which have been developed in recent times, such as better methods of doing *in vitro* studies? There are improved methods of homogenization of tissues, separation of cellular components, and extraction of water soluble enzymes. There are also, of course, the isotopes that are available and [illegible]

I [illegible]

poir [illegible]

deficient animals, and with methionine-deficient animals. You are all even more familiar with the profound results produced on the liver of such animals, so far as the fat content is concerned.

What are the metabolic reasons for the accumulation of fat in this tissue under these conditions? Is this because there is a failure to oxidize the fat? Is it because of increased synthesis or mobilization?

Some of these questions have probably been answered already by some of you. However, since we now have the important enzymes involved in the synthesis of the fats in aqueous solution, it should be possible to find out where the defect lies: what enzyme has been destroyed, if that be the case, and what cofactors are lacking. It seems to me that the time has come for an attack upon specific metabolic defects in this particular condition.

I shall speak chiefly on the synthetic aspect of the fatty acids and perhaps briefly on their oxidative fate primarily because there have

taille des ilots endocrines du pancréas et leur formule cellulaire *Ann. d'endocrinol.* 7, 57 (1946).

37. GREEN, D. M, NELSON, J. N., DODDS, G. A, and SMALLEY, R. E: Bilateral adrenalectomy in malignant hypertension and diabetes *J.A.M.A.* 144, 439 (1950).
38. HAIST, R. E, EVANS, M., KINASH, B, BRYANS, F. E, and ASHWORTH, M. A.: Factors affecting the volume of the islands of Langerhans. *Proc. Am. Diabetes A.* 9, 53 (1949)
39. KOBERNICK, S D, and MORE, R H.: Diabetic state with lipaemia and hydropic changes in the pancreas produced in rabbits by cortisone. *Proc. Soc. Exper. Biol & Med* 74, 602 (1950).
40. WARREN, S, and LE COMPTE, P. M: *The Pathology of Diabetes Mellitus* 3rd ed. Philadelphia, Lea & Febiger, 1952
41. POTTER, E. L: *Pathology of the Fetus and the Newborn* Chicago, Year Book Publishers, 1952.
42. MILLER, H C., JOHNSON, R D, and DURLACHER, S H: Comparison of newborn infants with erythroblastosis fetalis with those born to diabetic mothers. *J. Pediat* 24, 603 (1944)
43. GYÖRGY, P, and ROSE, C. S: Tocopherol and hemolysis in vivo and in vitro *Ann. New York Acad. Sc.* 52, 231 (1949)
44. ROSE, C S., and GYÖRGY, P.: Hemolysis with alloxan and alloxan-like compounds, and protective action of tocopherol *Blood* 5, 1062 (1950).
45. ———. Specificity of hemolytic reaction in vitamin E-deficient erythrocytes *Am. J Physiol* 168, 414 (1952)
46. GYÖRGY, P, COGAN, G, and ROSE, C S. Availability of vitamin E in the newborn infant *Proc Soc. Exper. Biol. & Med* 81, 536 (1952).
47. JOHN, H J Prediabetics: what becomes of them? *Am J Digest Dis* 17, 219 (1950).
48. GOMORI, G Pathology of the pancreatic islets *Arch Path* 36, 217 (1943)
49. ALLAN, F. N, BOECK, W. C, and JUDD, E S: Surgical treatment of hyperinsulinism *J A M A* 94, 1116 (1930)
50. POMPEN, A W M, JANSEN, C. A L, and DHONT, J: Adenoma of the islets of Langerhans and pregnancy *Acta med scandinav.* 124, 334 (1946)
51. HOWLAND, G, CAMPBELL, W R, MALTBY, E J, and ROBINSON, W L Dysinsulinism, convulsions and coma due to islet cell tumor of pancreas, with operation and cure. *J A M A* 93, 674 (1929)
52. FERNER, H Über die Entwicklung der *Langerhansschen* Inseln nach der Geburt und die Bedeutung der versilberbaren Zellen im Pankreas des Menschen *Ztschr. f mikr-anat Forsch.* 44, 451 (1938)
53. LAGUESSE, E Résultats éloignés de la résection du canal pancréatique chez le lapin *J de physiol. et de path gén* 13, 673 (1911)

fatty acids, and probably also of the carbohydrates. Acetyl coenzyme A is derived, too, from pyruvate as a result of the action of pyruvic oxidase, and so here we have a common meeting ground for both carbohydrate breakdown and fat breakdown.

The acetyl coenzyme A can condense with itself under appropriate conditions to form acetoacetyl coenzyme A. This has been worked out very beautifully in F Lipmann's (2) laboratory. There is an enzyme in liver which very actively and rapidly hydrolyzes the coenzyme A away from the acetoacetyl coenzyme A to produce acetoacetate Acetoacetate, in liver, represents an end product of metabolism It is slowly, and with difficulty, reactivated by the liver to form acetoacetyl coenzyme A, and so I have not put an arrow there pointing in the opposite direction There is a small amount of activation possible in the liver, but by and large the acetoacetate is relatively inert in the liver, a fact that is well known to all of you

The central compound, acetyl coenzyme A, may also be reutilized for resynthesis of long-chain fatty acids The arrow on the left, going upwards, indicates the possibility that the liver can utilize this two-carbon fragment for the resynthesis of fatty acids

I should like to summarize some recent work which has come to fruition in recent weeks after many years of study on the oxidation of the fatty acids by the liver (Figure 13) Not all of the individual steps have been rigorously proved, but I think there is enough evidence to indicate that the mechanism involved is probably correct, it involves an activation of long-chain fatty acids derived from triglycerides and phospholipids The reaction probably requires

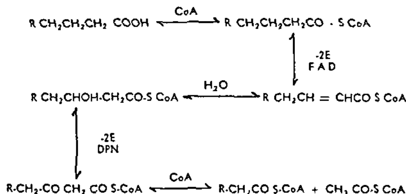


FIGURE 13

been, I think, some very important recent developments in th
Before doing so, however, I should like to call your atten
the framework within which I am going to talk Figure 12

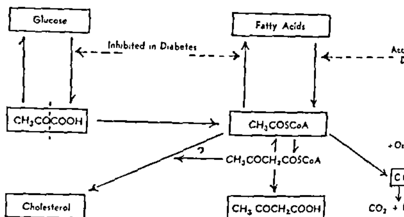


FIGURE 12

a very simple scheme which will serve to orient those of you
have not been keeping up with the work going on in this iso
metabolic, *in vitro* field.

Fatty acids can be, and are, oxidized by the liver by a p
that I think is now fairly well understood. The major pr
formed upon oxidation of fatty acids is probably largely a
coenzyme A, which contains an acetyl group attached to the
of coenzyme A This substance, I think, is probably the long-m
mysterious 2-carbon fragment that can be derived from fatty a
The oxidative process is not completely clear, that has not
settled. However, I think there is no doubt that the predom
product resulting from the oxidation of the long-chain fatty
in the liver is acetyl coenzyme A

The fate of acetyl coenzyme A is also reasonably well unders
This substance, as has been so beautifully demonstrated by S
and Ochoa (1), will condense with oxalacetate to form cit
This system has been isolated The enzyme involved has been p
fied, and in a solution of the enzyme with acetyl coenzyme A,
oxalacetate, citric acid can be formed

I think most of us are agreed that this represents probably
major pathway by which the acetyl portion of coenzyme A
oxidized to CO_2 and water Thus, the series of reactions thro
the Krebs cycle is probably the major pathway of oxidation of

fatty acids, and probably also of the carbohydrates Acetyl coenzyme A is derived, too, from pyruvate as a result of the action of pyruvic oxidase, and so here we have a common meeting ground for both carbohydrate breakdown and fat breakdown

The acetyl coenzyme A can condense with itself under appropriate conditions to form acetoacetyl coenzyme A This has been worked out very beautifully in F. Lipmann's (2) laboratory. There is an enzyme in liver which very actively and rapidly hydrolyzes the coenzyme A away from the acetoacetyl coenzyme A to produce acetoacetate Acetoacetate, in liver, represents an end product of metabolism It is slowly, and with difficulty, reactivated by the liver to form acetoacetyl coenzyme A, and so I have not put an arrow there pointing in the opposite direction. There is a small amount of activation possible in the liver, but by and large the acetoacetate is relatively inert in the liver, a fact that is well known to all of you.

The central compound, acetyl coenzyme A, may also be reutilized for resynthesis of long-chain fatty acids The arrow on the left, going upwards, indicates the possibility that the liver can utilize this two-carbon fragment for the resynthesis of fatty acids

I should like to summarize some recent work which has come to fruition in recent weeks after many years of study on the oxidation of the fatty acids by the liver (Figure 13). Not all of the individual steps have been rigorously proved, but I think there is enough evidence to indicate that the mechanism involved is probably correct, it involves an activation of long-chain fatty acids derived from triglycerides and phospholipids. The reaction probably requires

$$R\text{-}CH_2CH_2CH_2\ COOH \xrightarrow{CoA} R\ CH_2CH_2CH_2CO \cdot S\,CoA$$

$$\downarrow \text{-2E, FAD}$$

$$R\ CH_2CHOH\ CH_2CO\ S\,CoA \xleftarrow{H_2O} R\ CH_2CH = CHCO\text{·}S\,CoA$$

$$\downarrow \text{-2E, DPN}$$

$$R\ CH_2\ CO\ CH_2\text{·}CO\ S\,CoA \xrightarrow{CoA} R\text{·}CH_2CO\ S\,CoA + CH_3\text{·}CO\text{·}S\,CoA$$

FIGURE 13

ATP (adenosinetriphosphate) and coenzyme A to give a long chain acid which is combined through its carboxyl to the sulfur group of coenzyme A, a long-chain acyl coenzyme A. This activated fatty acid may now be oxidized (the enzyme involved is a flavoprotein) to an unsaturated acid which is still attached to coenzyme A. This alpha-beta unsaturated compound is hydrated to form the beta-hydroxy acid coenzyme A complex, which may then be oxidized, thereby losing two electrons, by a system requiring DPN, or coenzyme III, to form a beta keto acid, still linked to coenzyme A

At this point there is a cleavage which involves an enzyme that has been called thiolase and a second molecule of coenzyme A Here now is the beta oxidation that all of us have known for many years: a cleavage forming one molecule of acetyl coenzyme A and an acid which is now shorter by 2 carbons, but which is still attached to coenzyme A.

What has been accomplished by this? Two carbons have been broken off. We now have an acid shorter by two carbons, but this is in an activated state, and the process may now continue until all of the fatty acid has been cleaved into acetyl coenzyme A This we may call a fatty acid oxidizing cycle, if we like As I say, many points will need further investigation, but there is enough evidence now to convince most of us that this represents the way in which the liver certainly, and perhaps other tissues, oxidize fatty acids

In the time I have at my disposal I cannot pay tribute to all of the investigators involved in this study, but I might say that starting with the brilliant work of Munoz and Leloir (3) a number of years ago homogenates were first made that were capable of oxidizing fatty acids This was continued in the laboratories of Lehninger (4), and later Lipmann (2), Lynen (5), Ochoa (6), and Green (7) There were many others. The details of this particular mechanism could not have been worked out without these newer *in vitro* techniques of homogenizing, separating cellular components from such homogenates, extracting out the soluble enzymes involved in each of these steps, determining the cofactor requirements, and finally separating the enzymes to study each reaction individually, because it is only in this way that most intermediates can be made to accumulate This has been the secret, I think, of the success in this particular area.

Shorr Would you kindly repeat step 4?

Gurin We have a hydration of the alpha-beta unsaturated acid to form the beta-hydroxy acyl coenzyme A. This is quite similar to the standard metabolic reactions that we have seen in textbooks

for the last twenty years, the only difference being that this is all accomplished on intermediates attached to the coenzyme A molecule We are going through an alpha-beta unsaturated acid, a beta-hydroxy, and a beta-keto acid, but the only difference is that these are combined with coenzyme A and are not there as free acids This, of course, is one of the reasons why they have been difficult to identify. The other reason is that these enzymes are active metabolically and intermediates do not accumulate. It is only when we can purify the enzymes that we can have such intermediates accumulate. You will notice that Figure 12 has separate arrows indicating synthesis and oxidation This is only for the purpose of simplicity, and not to imply that the reactions may be totally different

This is one area in which there is some debate I think most investigators believe at the moment that the reactions involved in the oxidation of the fatty acids down to acetyl coenzyme A are really reversible, and that the acetyl coenzyme A can condense with itself by the same series of reactions to form long-chain fatty acids However, this has not yet been established

I will say this it has been possible to demonstrate in isolated enzyme systems that acetyl coenzyme A can form, or be converted into, butyryl coenzyme A In other words, 2 molecules of acetyl coenzyme A have condensed to form acetoacetyl coenzyme A, and then by a simple reversal of the reactions that I have shown in this figure, we obtain butyryl coenzyme A

Shorr How about propionyl?

Gurin. I believe it has been demonstrated that that will also condense

Shorr Has it? There is a very curious situation with propionic acid, at least in certain tissues Furchgott and I (8), working with smooth muscle of the rabbit intestine, showed that fatty acids would support the contractility of muscles which had been allowed to run down through the exhaustion of their carbohydrate stores during incubation in the substrate-free medium When we added butyric acid, contractility was resumed If we then added propionic acid, we inhibited the contractility due to butyric acid, and if we added more butyric acid, we could overcome the inhibition due to propionic acid We interpreted our experiments as indicating that beta-oxidation occurred in intestinal smooth muscle, and that propionic acid and the two carbon fatty acid fragments competed for the same enzyme surface When there was a preponderance of two carbon fragments, smooth muscle contraction went on but

when propionic acid predominated, contractility ceased, because the concentration of propionic acid prevented the two carbon fragments from getting on to the enzyme surface.

Gurin: As a matter of fact, Stadtman (9), I believe, demonstated with micro-organisms, this condensation of a 3-carbon with a 2-carbon fragment, and it is a rather similar type of condensation

If the process is truly reversible — and that means the enzymatic steps are the same in both oxidation and synthesis — there are a number of questions, of course, that are immediately raised For example, what decides which shall predominate: oxidation or synthesis? How do hormonal and nutritional disturbances affect the individual steps involved in this synthetic process?

I have been interested in the first question: what enables the synthetic process to go on, and what helps it go one way or the other. Some of the work that has been done by my colleagues bears on that particular point. We have been using primarily an extract of liver, which is particle-free, contains soluble cytoplasmic enzymes, and also an extract of mitochondria Thus, we have in this water-soluble system the enzymes extractable from the mitochondrial fraction obtained from the liver, plus what remained in the supernatant fraction.

Shorr. May we have, for the record, your method of making the extract?

Gurin: Dr F Dituri (10) and Mrs J. Warms (11) have done this Liver is homogenized in a phosphate buffer, in a Potter-Elvehjem homogenizer; subsequently, it is spun down at low temperature to remove cell debris and nuclei. At higher speeds, the mitochondria are brought down; they may then be washed, and the wash discarded. They may be treated with hypotonic solutions, or water, for a half-hour, with very gentle stirring. This seems to extract from the mitochondria the enzymes needed for synthesis After this treatment, the supernatant fraction is combined with this suspension of mitochondria in water and then spun at extremely high speeds in the ultracentrifuge until particle-free The resulting supernatant fluid is what we use for this biosynthesis. A bit later I shall mention some of the cofactors which are needed in that synthesis I might say that the same process is used to prepare an extract that will synthesize cholesterol from acetate or pyruvate

Figure 12 represents some of the reactions I talked about Acetyl coenzyme A, plus oxalacetate, gives rise to citrate. The other reaction involves 2 acetyl coenzyme A molecules combining to yield acetoacetyl coenzyme A The acetoacetyl coenzyme A may be re-

duced to beta-hydroxybutyryl coenzyme A, and subsequently to butyryl coenzyme A. This would be the pathway of synthesis of fat in the liver. Since there is an active enzyme which splits off coenzyme A from acetoacetyl coenzyme A, it would hydrolyze to give acetoacetic acid plus coenzyme A

TABLE IV

Incorporation of 1-C^{14} Acetate and 2-C^{14} Pyruvate into Long-Chain Fatty Acids by Particle-Free Extracts of Rat Liver

Substrate	Concentration	μMoles Incorporated
Acetate	35 μMoles	0 04
"	35 μMoles	0 04
Pyruvate	17 5 μMoles	0 14
"	35 μMoles	0 23
"	52 5 μMoles	0 38

Table IV shows some of the results one may obtain in a study of the water extracts taken from liver You will see that the incorporation of labeled acetate into fatty acids is appreciable, these experiments have been done with C^{14} labeled acetate and pyruvate.

Shorr No added coenzyme A?

Gurin We do not need to add coenzyme A, since there is a sufficient amount present Actually, the system does require coenzyme A but it has been difficult to demonstrate it We have used DPN, ATP, coenzyme A, and the incorporation into fat is remarkably stimulated by citrate, as I shall show later

You will note that the pyruvate is utilized much more efficiently than acetate I might say this is in decided contrast to previous results obtained with liver slices, both in our laboratory and in that of Konrad Bloch (12) By increasing the concentration of pyruvate

that carbohydrate is a much better source of fat than vinegar.

I might say, too, while I am on the matter, that such an extract prepared from pigeon liver is much more effective, so far as the synthesis of fat is concerned, than the rat extract The reason for

this is that we have a very strong acetate-activating system in pigeon liver I must remind you that when acetate is used as a substrate, it must first be converted to acetyl coenzyme A, a process which is difficult in the rat liver Lipmann (2) has studied this system very thoroughly. Pigeon liver does a much better job of converting acetate into long-chain fatty acids than rat liver. The isotope found in the long-chain fatty acids resulting from this incubation is distributed throughout the chain length, so I am quite certain that this represents a true synthesis, and not simply an incorporation in a limited part of the fatty acid molecule

If one makes the same kind of solution from the liver of an alloxan diabetic rat, for example, there is almost no lipogenesis at all from either acetate or pyruvate This, of course, is not too surprising There has been a good deal of work done in recent years which was started by Drury (13), and then Stetten (14), demonstrating a breakdown in the ability of diabetic animals to convert carbohydrate to fat. Brady and I (15) have done some work with liver slices which some of you may be familiar with, showing that the liver slices obtained from alloxanized rats, or depancreatized cats, lose their ability to convert radioactive acetate into long-chain fatty acids

We became interested in this question of how such extracts differ from normal extracts. An important clue came from the striking work of Baker *et al.* (16). Most of you will recall that he found that liver slices of diabetic rats, which had been pre-fed with fructose, were capable of synthesizing fat from lactate or acetate, but not from glucose. Lyon *et al.* (17), also demonstrated that liver slices obtained from fasted animals were relatively incapable of synthesizing fat Similar work was done by Van Bruggen *et al.* (18)

Thus, it seems pretty clear that the diabetic liver slice – and I refer to that from the alloxanized animal – is able to utilize fructose fairly well It is apparently able to glycolyze the fructose efficiently There was other evidence that lactate is fairly well oxidized by diabetic liver slices, and that pyruvate is just as well decarboxylated by diabetic tissue as it is by normal tissue There is a considerable body of evidence that fatty acids are rapidly oxidized in the diabetic state Thus, from this material, we may draw the following conclusions

a) Most of the glycolytic enzymes appear to be intact in the diabetic state

b) The oxidative enzymes appear to be intact; these tissues have the ability to activate fatty acids, and to oxidize such substances as lactate, pyruvate, and acetate

Chaikoff (19), as I understand it, interpreted his findings to mean that lipogenesis was somehow tied up with glycolysis As I interpret his experiments, this indicated to him that the fundamental block was somewhere before the glucose-6-phosphate level, whether it was at the hexokinase or the cell permeability level is of course not established He was not certain whether there was another block in the conversion of acetate to fat I believe at one time he did feel that such a block was involved.

Table V will indicate a few of the results obtained by Mr. Walter Shaw in our laboratory. Here is a water-soluble extract; there are no membranes involved I am simply reporting the counts per minute in the recovered fatty acid, and all of these experiments were done under exactly the same conditions You see that the fatty acid recovered contained anywhere from 60 to 100 counts per minute per mg of fatty acid. The diabetic solutions yield counts ranging from about 4 to 15 counts, in contrast to the much higher figure obtained with normal extracts If to this "diabetic solution" we add mitochondria obtained from the livers of normal rats, there is no stimulation We then added the supernatant fluid obtained from the livers of normal rats There was a very considerable stimulation in a number of cases Thus, it became clear to us that there was something in the supernatant fluid which was stimulating these "diabetic extracts," and which permitted them to increase the amount of fat capable of being synthesized When we added glycogen, a good effect was observed with control values of from 6 to 32, and from 13 to 50 Finally, hexosediphosphate had a very pronounced effect It practically brought lipogenesis back to normal Thus, simply by the addition of hexosediphosphate to a "diabetic solution," we could restore lipogenesis to normal, or very close to normal rates

You will see that when the "diabetic solutions" are supplemented with glucose, there is no effect. We have done this several times The addition of glucose to such extracts is without effect

If we add glucose-6-phosphate, we double or triple the incorporation The effect is not tremendous, but it is definitely significant Incidentally, if we add ATP along with the glucose-6-phosphate, we double the effect that we obtain with glucose-6-phosphate Finally, hexosediphosphate has again an enormous effect You will see that fructose – we have run only one or two experiments – has an effect which is greater than that of glucose. Glucose has no effect, and fructose is very definitely better Phosphorylated intermediates

TABLE V

Lipogenesis by Particle-Free Extracts of Alloxan-Diabetic Rat Liver

Preparation		Additions	Recovered Fatty Acids: Counts per min per mg Fatty Acid 1	Counts per min per mg Fatty Acid 2	Micromoles of Pyruvate Incorporated per 10 mg. Fatty Acid 1	Micromoles of Pyruvate Incorporated per 10 mg. Fatty Acid 2
1	Normal	None	82.0	99.6	0.18	0.21
	Diabetic	None	5.6	13.3	0.01	0.03
	Diabetic	Normal mitochondria	6.1		0.01	
	Diabetic	Normal supernatant	18.9	31.6	0.04	0.07
	Diabetic	Glycogen 50 mg	32.2	49.8	0.07	0.11
	Diabetic	Hexosediphosphate 11 µMoles	51.5	62.5	0.11	0.13
2	Diabetic	None	8.0	4.4	0.02	0.01
	Diabetic	Glucose 11 µMoles	7.8*		0.02	
	Diabetic	Glucose-6-phosphate. 11 µMoles	15.6	11.0	0.03	0.02
	Diabetic	Glucose-6-phosphate 11 µMoles + 1 mg ATP†		27.1		0.06
	Diabetic	Hexosediphosphate 11 µMoles		48.0		0.11
3	Diabetic	None	5.7		0.01	
	Diabetic	Fructose 11 µMoles	21.2*		0.05	
	Diabetic	Hexosediphosphate· 11 µMoles	50.2		0.11	

* These results have been confirmed several times † Adenosinetriphosphate

The complete system consisted of 4.5 ml of enzyme preparation, 0.01 M K citrate (final concentration), 54.5 µMoles of pyruvate-2-C^{14} (1.25 x 10^{6} counts per min mg C), plus the additions indicated below The total volume, 5 ml, gas phase, air, incubation time, 3 hr, temp 34° C

Reprinted, by permission, from Shaw, W, and Gurin, S Relationship of glycolysis to lipogenesis in aqueous extracts of liver *Arch Biochem* 47, 221 (1953)

are extremely effective It appears that glycolysis is necessary for efficient fat synthesis, but what the reason for this is, I do not know.

Shorr: Do you mean glycolysis or turnover?

Gurin: I do not know just what I mean by that. I think that we have active glycolysis, and the process has to be going on actively. Whether it is to furnish reduced DPN, I do not know. We need a source of hydrogen for this synthesis, but I do not know whether it will produce energy of the right kind All I can say is that if we add phosphorylated intermediates of glycolysis, lipogenesis proceeds in a solution which would otherwise be inert or relatively inert.

Shorr. There is some evidence which Dr. Furchgott and I reported that the turnover of high energy phosphate in viable skeletal muscle is equally rapid when muscle burns at a respiratory quotient (R Q) of 0.7, as when it burns at an R Q of 1 Therefore, phosphorylative processes must be going on in relation to fatty acid oxidation

Gurin: I have no doubt that in this case the rate of phosphorylation is limiting If we compare the effects of glucose, glucose-6-phosphate with and without ATP, and finally HDP, we reach this conclusion

Houssay The limiting factor is glucose-6-phosphate?

Gurin Yes, another point is that the same enzyme solution which utilizes glucose-6-phosphate for the stimulation of lipogenesis is unable to use glucose with or without the addition of insulin *in vitro*

Lillie. This is alloxan diabetes?

Gurin: Yes The same solution which can use glucose-6-phosphate apparently cannot utilize glucose, and it may be that we have here an indirect way of measuring the hexokinase reaction

I have interpreted all of this to mean that the inability to synthesis fat, which is associated with the diabetic state, may be a reflection of a diminished intracellular concentration of phosphorylated intermediates Whether or not this is true I cannot say, but I suggest it as a possibility Whether this also explains the phenomenon observed in fasting, I am not sure, but it seems like an attractive speculation at the moment I think it certainly needs further investigation

Table VI represents the cofactor requirements of this system Since we have a water-soluble system, it is rather easy to get rid of cofactors, but not by dialysis, surprisingly enough, because our enzyme system seems to be readily inactivated However, simple treatment with charcoal removes ATP and DPN, and if used sufficiently exhaustively will even remove a fair amount of coenzyme A What we do is simply to stir the enzyme solution for five or ten

TABLE VI

Cofactor Requirements for Lipogenesis by Aqueous Liver Extracts

System	Cofactors	Radioactivity cpm per mg Fatty Acid	
Normal Control	—		120
" "	DPN	52	140
Charcoal System	—	0	0
" "	DPN	9	21
" "	ATP	3	2
" "	Co A	2	0
" "	DPN, ATP	18	9
" "	DPN, Co A	129	340
" "	ATP, Co A	14	32
" "	DPN, ATP, Co A	213	500

TABLE VII

Lipogenesis from C^{14} Acetate by Aqueous Extract of Pigeon Liver

Conditions		Acetate Incorporated (μMoles)
Control		0.03
"	+ Citrate	4.77
"	+ *l*-Glutamate	0.33
"	+ Oxalacetate	0.70
"	+ α-Ketoglutarate	1.15

minutes with charcoal, centrifuge and filter it. If we do this three or four times, we can remove considerable coenzyme A. This is a procedure which has been utilized by a number of laboratories By adding back the coenzyme A, one can see that some of the lipogenic activity is restored. I am reasonably certain that the system also requires a flavoprotein. As yet, we have not been able to remove it.

Table VII illustrates the citric effect that I mentioned previously. In addition to the cofactors required for active fatty acid

synthesis, citrate has an enormous effect, much greater than that of alpha-beta glutaric acid, oxalacetate, and glutamic acid.

Shorr: Do you know why, or what the process involved is?

Gurin: I have not the vaguest notion.

Shorr: Have you tried versene?

Gurin: We have tried the versenes, which do not do this. I do not believe this is a calcium-complexing effect, although calcium is quite inhibitory to this process. However, I must confess that I do not know what the actual mechanism of the citrate effect is. We have attempted to study that in some detail.

TABLE VIII

Effect of Citrate upon Aqueous Extract of Pigeon Liver

Acetate Incorporated into	+ Citrate (μMoles)	− Citrate (μMoles)
Fatty Acids	10.00	0.02
Cholesterol	0.00	0.00
Citrate	0.01	0.01
CO_2	0.40	0.80
Acetoacetate	3.00	7.00
β-hydroxybutyrate	0.70	3.00

Table VIII demonstrates what happens in the absence and presence of citrate when this water-soluble system — which, incidentally, is obtained from pigeons — is incubated with radioactive acetate. We divide the solution of radioactive acetate into two halves. To one half we add citrate, to the other we do not. We have tried to determine the distribution of the isotope among the components that you see. We note that in the presence of the citrate, much less acetate is converted into acetoacetate and β-hydroxybutyrate, than is the case when there is no citrate left. There is simply more acetate converted into ketone bodies in the absence of citrate than in its presence. Those of you who have studied antiketogenic substances for years, will be somewhat amazed to see a case of antiketogenesis described in an aqueous solution in a test tube.

Shorr: Does fluoracetate have any effect on this reaction?

Gurin: We have not yet studied it, but we should do so. These results do not account completely for the stimulating effect of citrate on fatty acids. On a quantitative basis it does not account for it, so I still do not know what the reason for this is

Shorr: What happens to the citrate? Have you quantitated it?

Gurin: Yes, we have in one experiment. Actually, there is very little disappearance. You see, we do not have the Krebs cycle here We certainly do not have the complete cycle, or the cytochrome oxidase system Thus, it is relatively inert.

Gyorgy: Why did you exclude the chelating effect of citrate?

Gurin: We assumed the effect of citrate was to tie up the calcium, and so we did some experiments with versene in different concentrations; we could come to no conclusions whatsoever.

Shorr. What pH did you run at?

Gurin· I do not recall what the pH's were, we ran it at several pH's. Most of them, I think, were between 7 and 7.8

Hartroft: I wonder if the stimulating effect of citrate on fatty acids might not be explained in this way. With reference to Table VIII, if citrate were added, would there not then be so much present that the reaction could not progress further in that direction?

Gurin The difficulty is that the equilibrium is far over on the side of the citrate, and it is practically impossible to drive it back in the opposite direction. Even if we piled up the citrate, I doubt if we could form much acetyl coenzyme A from the citrate

Hartroft I was wondering not so much whether the reaction might be reversed, as whether the addition of citrate would simply halt the flow.

Gurin: If what I said is true, then I think it is also true that it would not prevent the formation

I should like to speak of the few studies made by Dr. J L. Rabinowitz (20) on the biosynthesis of cholesterol from acetate and pyruvate in a similar aqueous enzyme system The difficulties have been much greater here. For years, everyone working in the field has attempted to make a cell-free suspension of liver that would synthesize cholesterol and has always failed Dr. Bucher (21), at the Massachusetts General Hospital, succeeded by using a loose-fitting Potter-Elvehjem homogenizer for a short period of time She discovered that this resulted in a suspension which she could show to be relatively cell-free, and which was rather efficient at converting labeled acetate into labeled cholesterol She very kindly informed me of this With our experience in making the

water-soluble system for fatty acid synthesis, it did not take us very long to develop a completely water-soluble particle-free system that would do the same thing, starting with homogenates as she prepared them We succeeded in doing this with the rat, but failed with the pigeon, which is a surprise to me, because I had always thought that pigeon liver could do anything However, it apparently cannot synthesize cholesterol when handled the way we treat rat liver. The incorporation into cholesterol was not as great as that obtained with fatty acid, but was nevertheless quite significant.

TABLE IX

Biosynthesis of Cholesterol by Aqueous Extract of Rat Liver

Substrate	Amount (μMoles)	Substrate Incorp (μMoles)
1–C^{14} Acetate	10	0 0012
"	10	0 0010
2–C^{14} Pyruvate	10	0 0150
"	10	0 0120

Table IX gives us an idea of the magnitude It is rather small, but we get counts of the order of 100 to 300 per mg. of cholesterol This, of course, depends upon the specific activity of the substrate that we start with, whether it be acetate or pyruvate It is certainly appreciable We can again see that pyruvate serves as a better source of carbon for cholesterol than acetate We will remember that acetate can be converted to acetoacetyl coenzyme A with difficulty, whereas pyruvate can be easily converted

We have also done some preliminary work on the distribution of the isotope in the resulting cholesterol We were naturally worried that we were simply studying an incorporation of labeled pyruvate or acetate into a small portion of the cholesterol, that a little fragment was slipping in and out, and that we were not really studying the biosynthesis of cholesterol. There are indications that there is about an equal distribution of isotope between the side chain, the nucleus, and the angular methyls Thus, I think it is probably fair to conclude from this that the enzymes present for the complete synthesis are there, although they may not be functioning at the most efficient rate After all, this is not truly physio-

logical, but nevertheless the enzymes are there, and I think we are actually studying the synthetic process.

The cofactors required for this system are rather similar to those of the fatty acid synthesizing system. We need DPN. Instead of just ATP, we may use any one of the adenosine phosphates, that is, adenylic acid, ADP, or ATP. Coenzyme A is required, and probably also flavoprotein, although we have not proved this The solution utilizes a small amount of oxygen, and in the absence of the cytochrome system, I assume it means that there is some flavoprotein involved which is utilizing some of the oxygen. We have invariably found a small uptake of oxygen with these solutions

So far as other precursors of cholesterol are concerned, not very much is known. We have been studying other precursors, such as acetoacetate. I think liver slices and whole liver can utilize labeled acetoacetate for cholesterol synthesis, but the water-soluble system is unable to do so. I presume this means that this system has lost whatever little ability it did have to activate acetoacetic acid to form acetoacetyl coenzyme A. Thus, we have been unable to incorporate acetoacetate into cholesterol.

This, of course, like so many other projects, leaves more questions unanswered than it answers We have been interested, first of all, in finding out how to prepare solutions that contain all of the enzymes necessary for a complex series of reactions, how they work, what cofactors they require, and what substances they make as intermediates After this, of course, there are the physiological problems How much of this is produced, how much of that is produced? Are these demonstrable reactions truly major reactions of interest to the physiologist, or are they not? This now becomes a matter of reaction rates, equilibria, removal of products, and so forth. Such factors, I think, decide whether more fat shall be made, more carbohydrate converted to fat, more fatty acid oxidized, or more cholesterol synthesized. It is perhaps in that area that all of these complex questions dealing with the role of hormones, for example, need to be studied. I am sure that that aspect of the problem will be investigated, and will certainly be discussed for many years.

Shorr Could I ask the basis for your statement that pyruvate is used just as well by the diabetic?

Gurin If we study, for example, the amount of pyruvate that goes to form acetoacetate, and can produce this in our systems, we shall see no difference. However, it is in a limited system Or if we study the acetylation reactions, we shall also find not too

much difference. I am very glad you brought this to my attention, because it illustrates once again how important it is to define whether we are talking about a limited system, or the whole animal.

Shorr: At one time, I made a comparative study (22) of the ability of certain diabetic tissues to utilize pyruvate, as measured by the increase in oxygen consumption and the respiratory quotient, and very striking differences were observed. In all the tissues studied, the normal tissue had a greater stimulation of oxygen consumption with pyruvate than did the diabetic; the respiratory quotient was curiously enough higher in the diabetic than in the normal tissue. If I recall correctly, it was most striking in the diabetic heart. There I obtained a quotient of 1.34 in the normal, and 1.20 – the theoretical one for the oxidation of pyruvic acid – in the diabetic tissue. With the Houssay animal, both the stimulation of respiration by pyruvate and the respiratory quotient returned to normal.

The question is: what is left behind in the diabetic? Do we have a system that is lacking some factor that is not a component of the essential enzyme systems? There, I think, we must not draw any conclusions unless they are confined to the specific tissue under study. For example, we cannot increase the anaerobic glycolysis of skeletal muscle of a diabetic by the addition of glucose. However, we can profoundly increase the anaerobic glycolysis of heart muscle of a diabetic by addition of glucose. Obviously, not all tissues have the same qualities.

Gurin: I might say that in work with labeled lactate, or pyruvate, going to CO_2, there is good evidence that there are unimpaired enzymes to oxidize those substances in diabetic tissue, particularly liver, as compared with normal.

Shorr: And yet, with some tissues, as the viable slice or strip, the response to lactate is better in the normal tissue. It may be that as we reduce activities to a certain level in specific types of extracts, and these differences which depend on organization are all blended, just as, for example, when we take out a brain and do respiratory measurements under the most optimal conditions *in vitro*, we have a reduction by certainly 95 per cent of the over-all rate of oxygen consumption which we cannot, to any real degree, elevate towards what prevails in the body.

Gurin: I do not like the idea of saying that it can be demonstrated that pyruvate forms acetoacetate quantitatively in washed mitochondria. Of course, this is a washed system, it is free of dicarboxylic acids. If we add the dicarboxylic acids, we do not get

obtain a deficiency of acetyl coenzyme A, and a diminution of some of these synthetic processes in which acetyl coenzyme A is utilized as a precursor. I think this is an area which is going to be studied very actively in the next few years, but I do not know any specific instances of studies of deprivation of some of the sulfur-containing acids.

Hanger. Suppose we made a water extract of a choline-deficient liver. Would we have any way of calibrating enzymatic activity in terms of the normal liver?

Best: We could easily estimate the coenzyme A, could we not

Gurin: Using labeled acetate or pyruvate, we could find out whether it synthesizes fatty acids, and if so, whether it oxidizes fatty acids? One could then introduce fatty acids Does it convert them to acetyl coenzyme A, or to acetoacetate, in this aqueous solution? In other words, what is the nature of the block, and what part of the mechanism is involved?

Hanger: Could we make any quantitative comparison between the two masses of tissue?

Gurin. I think all we could do, on a statistical basis, would be to compare what we obtain with an extract from a normal liver and the liver of a choline-deficient animal

Best. Dr. Rosenfeld, in our laboratory, did this for coenzyme A and instead of deficiency in the choline-deficient animal, as we guessed, there was actually an apparent increase.

Kosterlitz: I wonder whether Dr. Gurin's findings may explain some observations which go back as far as the preinsulin era. At that time, fructose was used therapeutically. It was found that it was better retained than glucose, and also had a very definite antiketogenic effect. The same holds for galactose. Both fructose and galactose are readily assimilated. That is to say they disappear from the blood stream as rapidly as in nondiabetic persons, indicating that the phosphorylating mechanism is not seriously damaged. Does that perhaps tie up with your findings that fructose improves lipogenesis? It might be very interesting to see whether galactose would have a similar effect, and perhaps also sorbitol, which is antiketogenic in human diabetes.

Gurin We have not studied sorbitol and galactose. I hope the fructose effect we obtained clinically is explainable on that basis. It has been established experimentally that in diabetic animals, at least, there is an ability to phosphorylate fructose, and these extracts can be stimulated, to some extent at least, by fructose. Just how this is being accomplished, of course, I do not know. One of

the reasons is that we do not yet know the many steps involved in reversing the whole series of oxidative steps. We have to supply energy and hydrogen. Presumably this is reduced DPN and reduced flavoprotein. The amazing thing to me is to find an extract that will do this at all A year ago I predicted that no one would synthesize cholesterol in a cell-free system, but now we are doing it in our own laboratory

Shorr. As regards fructose, I think we also have to bear in mind the question "with respect to what?" For example Loebel in 1925 (24) showed that fructose was oxidized by gray matter of the brain, but glycolysis did not take place

Kosterlitz: Of course, when fructose or galactose is phosphorylated, we obtain hexosediphosphate

Gurin. I wonder what the evidence is.

Shorr: I think direct lactic acid determinations as well as measurements of CO_2 production were made As far as I know the findings have never been contradicted

Neefe In states of advanced liver insufficiency, there has been some interesting evidence that increases in the concentration of alphaketoglutaric and pyruvic acids are found in the blood Have you any idea where the breakdown might be under those circumstances?

Popper. Increases, not only of ketoglutaric acid, but also of citric acid have been found (25,26).

Gurin: I have no logical explanation for that. I could offer suggestions, but would have no real evidence to base them on

Shorr Don't we have to ask ourselves about citric acid metabolism in the whole organism? Would we expect, offhand, that citric acid given to an animal would appear quantitatively as glucose? That is what happens ordinarily when we give citrate by mouth We recover it as glucose, or glycogen, in the liver, and then it subsequently comes out as glucose in the blood In terms of the whole organism, we thus have the citric acid cycle that we know However, there is also another citric acid pathway, and the large amount reappearing in the urine as glucose makes us wonder what we know about this other mechanism

Gurin Perhaps this is oversimplifying it a bit on my part.

Shorr We are all doing that

Gurin I am a little hesitant about saying this, but we all know that the carbons which go into citrate are not the ones that come out as glucose What we are doing is comparing something else In other words, the citrate can still go through the cycle, but something

else that might have entered into the mechanism has been pushed out, so that an equivalent amount of carbon comes out in the form of glucose.

We did some experiments with labeled lactate a few years ago, with phlorhizined animals, which indicated the same thing We can put in lactate and label it, but it is not those carbons that come out as glucose; it is some other nonradioactive or nonisotopic carbon

Shorr: Is this in an animal that has very little or no glycogen in its liver?

Gurin: Yes, I think the problem is largely one of masses It is a matter of equilibria, as I was saying before. The physiological problem is to ascertain how much of this or that is made, and how much is oxidized. This is what we can see and measure.

Best: I should like to have Dr. Houssay's and Dr. Gurin's reactions to this question. According to the paper by Brady, Lukens, and Gurin (27) on the lipogenesis of liver slices in the diabetic animal, you took out the pituitary and made a Houssay animal, and the lipogenesis returned. In the Houssay animal, the metabolic rate is what, Dr. Houssay? Is it 20 or 30 per cent lower than normal?

Houssay: It is lower than in the pancreatectomized animal It can be lower than the normal, yes

Best It interested me very much that the lipogenesis came back

Houssay. In my dogs there was about a 20 per cent decrease of the metabolic rate, which is not very much In the rats it is more definite, from 30 to 40 per cent.

Best. Thus, the metabolic activity of the tissue, let us say, was decreased by removal of the pituitary, and yet, when Dr Gurin and his colleagues studied the lipogenesis, it was at the normal rate

Gurin: At somewhere near the normal rate. On a quantitative basis, I prefer not to say unequivocally that we have a normal rate

However, I think this is important in considering what type of metabolism is going on, Dr Best. In the depancreatized animal, we are presumably having a tremendously accelerated oxidation of fatty acids In the Houssay animal, as I understand it, this is diminished, and we do not have the rapid oxidation of fatty acids On the other hand, we may have increased phosphorylation of carbohydrate

I think it is a matter of what kind of metabolism is going on. If we have, for example, a higher intracellular concentration of phosphorylated intermediates for glycolysis – I am looking at Dr Shorr when I say this, because I do not know what I mean by this

term — we might have better lipogenesis I think that may offer a reasonable explanation.

Houssay. In the whole rat, after hypophysectomy, there is a decrease of fat, because they eat very little; but after forced feeding they develop a large quantity of fat, more than the controls who receive the same quantity of food One important fact is, that under some emergency condition in which a normal animal catabolizes a great deal of fat, the hypophysectomized animal is apparently unable, after pancreatectomy to increase ketonuria After phlorhizin, they have also less ketonuria, the production of ketone bodies is very definitely diminished in the hypophysectomized animals That is one basic fact

Shorr. Is this in the hypophysectomized and pancreatectomized animals, or is it just in the hypophysectomized animals?

Houssay: In the hypophysectomized animals, as compared with the normal, and especially in those hypophysectomized and pancreatectomized, as compared with the depancreatized animals.

Shorr. Could some of it be due to the factor of mobilization?

Houssay: Yes, apparently the mobilization is lower

Gurin. Dr. Houssay, did I understand you to say that hypophy- [illegible]

[illegible] away the pancreas and the hypophysis, the animal has very little ketonuria in comparison with an animal without the pancreas and with the hypophysis Thus, hypophysectomy very much diminishes the production of ketone bodies This is one of the typical facts about the animal deprived of hypophysis

Gurin This fits in with the idea of increased lipogenesis, when the pituitary is removed in addition to the pancreas.

Houssay What is your explanation for the decreased catabolism of fat in the hypophysectomized animal?

Gurin The decreased oxidation of fatty acids?

Houssay Yes

Gurin I cannot answer that, Dr Houssay, I think we are now learning that one metabolic process influences another I believe glycolysis is an important process, particularly where synthetic processes are concerned It has been found to be true in the case of *in vitro* studies involving protein synthesis. Chaikoff (16) and we, ourselves, have proved this is true in the case of fatty acid synthesis Thus, I believe glycolysis plays a more important role than we have hitherto believed

Houssay: Yes, but in the hypophysectomized animal, we easily obtain deposition of fat, but not of protein, in spite of the fact that glycolysis is useful for both reactions.

Shorr: What about the glycogen content of the liver?

Houssay: It is high after a feeding. However, very soon the decrease during fasting is more marked than in the normal animals.

Shorr: It is after feeding that they make fat and store it.

Houssay: Yes

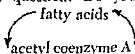
Best. They do not eat very much.

Gurin: The experiments which we published were made on a very small series of hypophysectomized animals.

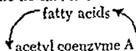
Houssay: The forced feeding is very important in the hypophysectomized animal.

Lundsgaard: Dr. Gurin, do you think it possible that a block in the synthesis of fatty acids from acetyl coenzyme A might be responsible for the condition which is met in a liver pouring out large amounts of ketone bodies? If not, the oxidation of fatty acids down to acetate must be increased considerably, as, according to my observations, the degradation of acetate following the "citrate route" is of exactly the same order of magnitude as in a liver with only a small output of ketone bodies.

I may also put the question: Do you think that the cycle



proceeds normally at a considerable rate, so that a blockade in the synthesis would result in an accumulation of acetoacetate, accompanied by an oxidation at normal rate of acetate via the Krebs cycle? Here we have to take into consideration the fact, which I have mentioned previously, that if we judge the rate of the breakdown to the acetate stage from the carbon dioxide output plus the ketone body formation, it is four to five times the rate which we see in the normal liver That is to say that the cycle



should proceed at a considerable rate to account for the formation of ketone bodies, simply due to a block in the resynthesis of acetyl coenzyme A to fatty acids I should imagine that it would be a rather expensive pattern of metabolism, if fatty acids were continually, at such a rate, broken down to acetic acid to be resynthesized into fatty acids

Gurin. Dr. Lundsgaard, in the past I have postulated just such a mechanism, because it seemed to me at one time that there was evidence that the pathway of synthesis might be different from that of breakdown. For example, in the case of the liver slices obtained from an alloxan diabetic animal, apparently oxidation was proceeding at an accelerated rate, and yet, if we put in labeled acetate or labeled pyruvate, there was very little incorporation into fatty acids. Thus, it seemed to me there was a chance that this was not a truly reversible process

Further, under anaerobic conditions, we can to some extent, although not completely, diminish fatty acid synthesis. So we have a second condition where we have synthesis of fatty acid and relatively little oxidation. Both of these factors together led me to believe, at one time, that the pathway of synthesis must be different from that of breakdown Thus, I put the two arrows far apart.

In view of the recent work on the reversible enzyme systems, my own feeling is that those two arrows should be brought together, and that this is probably a reversible system The factor which decides the way it will go is perhaps dependent upon the availability of reduced DPN as a source of hydrogen for this synthesis, high-energy phosphate, or something else However, I think most biochemists would believe, today, that the process is truly a reversible one, and the direction that it takes is dependent upon other factors, such as how much hydrogen is available to use in reducing all of these acetyl coenzyme A molecules which must condense to form a saturated long-chain fatty acid A lot of hydrogen must be supplied, and much energy I think the answer probably lies in sources of hydrogen and energy, rather than that there are two separate pathways

The rate with which the resulting acetyl coenzyme A is oxidized or converted into acetoacetyl coenzyme A, represents another unknown area It will certainly be dependent, to some extent, upon the availability of the citric acid condensing enzyme, and how much oxalacetate is available to make citrate I should suspect that undoubtedly, in some instances, this is a limiting factor. This oxalacetate must be derived from carbohydrate and pyruvate, by carboxylation

Lundsgaard. That should be a limiting factor for the oxidative degradation of acetate down to carbon dioxide

Gurin This is correct

Lundsgaard If this reaction proceeds at a normal rate, and at the same time, there is a largely increased formation of ketone

Houssay: Yes, but in the hypophysectomized animal, we easily obtain deposition of fat, but not of protein, in spite of the fact that glycolysis is useful for both reactions

Shorr: What about the glycogen content of the liver?

Houssay: It is high after a feeding. However, very soon the decrease during fasting is more marked than in the normal animals

Shorr: It is after feeding that they make fat and store it.

Houssay: Yes.

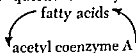
Best: They do not eat very much.

Gurin: The experiments which we published were made on a very small series of hypophysectomized animals

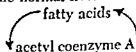
Houssay: The forced feeding is very important in the hypophysectomized animal.

Lundsgaard: Dr. Gurin, do you think it possible that a block in the synthesis of fatty acids from acetyl coenzyme A might be responsible for the condition which is met in a liver pouring out large amounts of ketone bodies? If not, the oxidation of fatty acids down to acetate must be increased considerably, as, according to my observations, the degradation of acetate following the "citrate route" is of exactly the same order of magnitude as in a liver with only a small output of ketone bodies.

I may also put the question: Do you think that the cycle



proceeds normally at a considerable rate, so that a blockade in the synthesis would result in an accumulation of acetoacetate, accompanied by an oxidation at normal rate of acetate via the Krebs cycle? Here we have to take into consideration the fact, which I have mentioned previously, that if we judge the rate of the breakdown to the acetate stage from the carbon dioxide output plus the ketone body formation, it is four to five times the rate which we see in the normal liver That is to say that the cycle



should proceed at a considerable rate to account for the formation of ketone bodies, simply due to a block in the resynthesis of acetyl coenzyme A to fatty acids I should imagine that it would be a rather expensive pattern of metabolism, if fatty acids were continually, at such a rate, broken down to acetic acid to be resynthesized into fatty acids.

Gurin. Dr. Lundsgaard, in the past I have postulated just such a mechanism, because it seemed to me at one time that there was evidence that the pathway of synthesis might be different from that of breakdown. For example, in the case of the liver slices obtained from an alloxan diabetic animal, apparently oxidation was proceeding at an accelerated rate, and yet, if we put in labeled acetate or labeled pyruvate, there was very little incorporation into fatty acids Thus, it seemed to me there was a chance that this was not a truly reversible process

Further, under anaerobic conditions, we can to some extent, although not completely, diminish fatty acid synthesis So we have a second condition where we have synthesis of fatty acid and relatively little oxidation Both of these factors together led me to believe, at one time, that the pathway of synthesis must be different from that of breakdown Thus, I put the two arrows far apart.

In view of the recent work on the reversible enzyme systems, my own feeling is that those two arrows should be brought together, and that this is probably a reversible system. The factor which decides the way it will go is perhaps dependent upon the availability of reduced DPN as a source of hydrogen for this synthesis, high-energy phosphate, or something else However, I think most biochemists would believe, today, that the process is truly a reversible one, and the direction that it takes is dependent upon other factors, such as how much hydrogen is available to use in reducing all of these acetyl coenzyme A molecules which must condense to form a saturated long-chain fatty acid A lot of hydrogen must be supplied, and much energy. I think the answer probably lies in sources of hydrogen and energy, rather than that there are two separate pathways

The rate with which the resulting acetyl coenzyme A is oxidized or converted into acetoacetyl coenzyme A, represents another unknown area It will certainly be dependent, to some extent, upon the availability of the citric acid condensing enzyme, and how much oxalacetate is available to make citrate I should suspect that undoubtedly, in some instances, this is a limiting factor This oxalacetate must be derived from carbohydrate and pyruvate, by carboxylation

Lundsgaard That should be a limiting factor for the oxidative degradation of acetate down to carbon dioxide

Gurin This is correct

Lundsgaard If this reaction proceeds at a normal rate, and at the same time, there is a largely increased formation of ketone

bodies, I think it must probably be due, either to a block in the resynthesis of acetate, or an enhancing effect on the primary steps of the oxidative breakdown, to acetyl coenzyme A.

Gurin: I think so. This may be a naive explanation, but has been confirmed, I think, by much experimentation. If the amount of acetyl coenzyme A is increased, as it may be in the diabetic state, it overpowers the amount of oxalacetate available for condensation to form citrate. The excess, I suppose, must follow this course and perhaps some becomes cholesterol, too. Of course, I am speculating I am not sure I have answered your question, Dr. Lundsgaard

Lundsgaard: Oh, yes, I think so, because as far as I understand you, you suggest that the oxidation of the fatty acids, for some reason or other, may be enhanced, without any change in the oxidation to carbon dioxide.

Gurin: Via the Krebs cycle. However, it is an increase in the breakdown of fatty acids. Is that what you mean?

Lundsgaard: Yes, the primary breakdown of acetyl coenzyme A You do get greatly increased acetoacetate production However, we cannot say that the acetoacetate formed represents the acetyl coenzyme A that might normally have gone back to fatty acids

Gurin: I believe Dr Sidney Weinhouse (28) has some experiments with labeled fatty acid. They show that there is increased breakdown of the fatty acids in liver slices of diabetic animals I believe this is correct

Hartroft: If there is increased breakdown of the fatty acids to acetyl coenzyme A, why is there a fatty liver in diabetes?

Gurin. Transportation and mobilization is probably the answer I do not believe it could be due to increased synthesis, because that is apparently diminished in the diabetic state

Hartroft: Is it because the oxidation still cannot keep up with the rate at which fat is brought to the liver in diabetes?

Best. There is a tremendous loss from the depots, as you know

Madden Is it true that sulfur is lost from the acetoacetyl coenzyme A?

Gurin. The sulfur stays there Lynen (29) did brilliant work on the isolation of acetyl coenzyme A.

Neefe How does one measure that?

Gurin There are various ways of attempting to get at it: It will react with hydroxylamine We can make acetyl phosphate from acetyl coenzyme A, and acetylhydroxamic acid by the reaction of acetyl coenzyme A with hydroxylamine. Lipmann (2) has been studying active acetate for many years, and there are several re-

actions that he has worked out for the estimation of acetyl coenzyme A. It is a rather labile substance, but just stable enough to work with.

Neefe. Can it be measured in blood or serum?

Gurin: I do not know how much is there, but I think the amounts are extremely small. The sources have been, I believe, liver and yeast. Lynen isolated it from yeast.

Lillie: How far can these results on the alloxan diabetic liver be transferred to, say, the pancreatectomized, phlorhizin, or human spontanous diabetic liver?

Gurin We have done some work with liver slices from the depancreatized cat, and they behave just the way the liver slices of the alloxanized rat do Thus, at least in those two instances, there is rather good correlation In both cases, there is a failure of lipogenesis.

Best. I think one of the main pitfalls with alloxan diabetes, is that so frequently there is a little insulin left. When the whole pancreas is removed, a good many digestive enzymes are lost, but we do not have to worry about the insulin that may remain Even in quite severe alloxan diabetes, some insulin may still be produced.

Hartroft. Is there a recognizable histological abnormality in the diabetic liver, other than that of an abnormal accumulation of stainable fat?

Popper Several changes have been reported Rossle (30) described as sclerosis an increase of intralobular collagen Quite frequently collagen membranes can be demonstrated in diabetic livers to enforce the reticulum framework. Furthermore, the Kupffer cells are sometimes markedly enlarged, which, however, is not characteristic for diabetes. More typical is their appearance under the fluorescent microscope, in that they are heavily loaded with fluorescent vitamin A, whereas the liver cells reveal very little (31) Finally, in addition to fat, glycogen is found in higher amounts in the diabetic liver than is otherwise seen in the cadaver liver after the usual agonal period All that holds true mainly for non- or poorly-compensated diabetes, and therefore these changes are now infrequently found Grossly, such glycogen-rich livers have a rosy hue which may be due to the increased glycogen content, as has already been assumed many years ago It is a typical picture which, however, is present only in some of the patients dying in diabetic coma

Neefe Is there not some change in the nuclei?

Popper: Due to glycogen deposition, the nuclei appear ballooned in diabetes, especially in the periportal zone. This has been called glycogen degeneration of the nuclei. It occurs, however, in a variety of conditions including passive congestion (32). It is more regularly found in diabetes than in any other condition and has been seen also in liver biopsy specimens (33). Repeatedly we noticed this picture in biopsy specimens in the absence of congestion and subsequently learned that the patients had diabetes.

Neefe: Dr. William Ehrich called our attention to it on some of our biopsies and explained that there were changes in the nuclei I did not recall what they were

Gurin: The only difference we have observed between diabetic and normal tissue is localized in the mitochondria, i e, the mitochondrial fraction We have observed repeatedly, after obtaining this fraction from livers of diabetic rats, that these mitochondria apparently do not lyse in water as readily as those from normal livers. When we compare the two, there is an immediate turbidity as soon as we add the water to the mitochondria of the normal liver; it becomes milky. It is quite apparent that they are breaking up, or that something is being extracted. The mitochondria of the diabetic livers, on the other hand, appear to be considerably more resistant. This worried us for some time, because of our conviction that the enzymes necessary for the synthesis of fat reside in the mitochondria. So we made rather lengthy extractions Of course, we had fortunately worked with whole homogenates and livers, which confirmed the fact that the whole tissue could not synthesize fat, we have noticed that difference frequently. What that is due to, I do not know, and whether they would show differences if properly stained, I have not the faintest notion

Popper Fat stains quite often produce artefacts, as I am sure Dr. Lillie will confirm. I should rather not commit myself as to the staining of mitochondria in this condition and would like to hear what Dr Lillie thinks about it.

Lillie. I am not too happy about mitochondria. I think if we could obtain perfectly fresh material from liver biopsy, supervital methods could be used successfully. The older hematoxylin methods, of course, were not too specific.

Gurin It seems to me it is worth studying. The thing that impresses me is that this is the structure of the cell that respires and carries out oxidative processes. It produces the CO_2 and uses the oxygen This particle is a perfect little chemical factory, and I believe it is going to be the center of interest for a long time to come

Lillie: I think perhaps electronmicrograph studies will be increasingly directed to the mitochondria.

Gyorgy. Dr. Madden asked about coenzyme A and its possible connection with deficiency of sulfur-containing amino acids. Last spring, at the New York Academy of Medicine meeting, Dr. R. E Olson, of Pittsburgh, claimed that in dietary liver injury, leading to massive necrosis of the liver, which is based on a double deficiency of sulfur-containing amino acids and vitamin E, the coenzyme A is very much reduced, and further, that vitamin E prevents the reduction of production of coenzyme A.

Gurin: It would not be surprising if there were such a relationship, because the thiolethylamine group in the coenzyme A molecule undoubtedly arises from one of the sulfur-containing amino acids

Best. In these conferences we have debated again and again everything we know about the action of choline. I think all of us are agreed that we have not been impressed by the claims that insulin has anything to do with arteriosclerosis The data of Steiner, Kendall, and Morrison, recently reviewed by J D Davidson (34), indicate that there is no true connection We have recently been working in our own laboratory, the Banting and Best Department of Medical Research, with severe choline deficiency Dr Hartroft, will you outline this for us?

REFERENCES

1 STERN, J R, and OCHOA, S Enzymatic synthesis of citric acid I Synthesis with soluble enzymes *J Biol Chem* 191, *161 (1951)*

2 LIPMANN, F Biosynthetic mechanisms *Harvey Lect* 44, *99 (1948 49)*

3 MUNOZ, J M, and LELOIR, L F Fatty acid oxidation by liver enzymes *J Biol Chem* 147, 355 *(1943)*

4 LEHNINGER, A L A quantitative study of the products of fatty acid oxidation in liver suspensions *J Biol Chem* 164, *291 (1946)*

5 LYNEN, F Functional group of coenzyme A and its metabolic relations especially in the fatty acid cycle *Federation Proc* 12, *683 (1953)*

6 OCHOA S Biological mechanisms of carboxylation and decarboxylation *Physiol Rev* 31, 56 *(1951)*

7 MAHLER, H R Role of coenzyme A in fatty acid metabolism *Federation Proc* 12, *694 (1953)*

8 FURCHGOTT, R F, and SHORR, E Sources of energy for intestinal smooth muscle contraction *Proc Soc Exper Biol & Med* 61, *280 (1946)*

9. STADTMAN, E R, and BARKER, H. A.: Fatty acid synthesis by enzyme preparations of clostridium kluyveri VI Reactions of acyl phosphates *J. Biol Chem* 184, *769 (1950)*
10 DITURI, F, and GURIN, S · Lipogenesis by homogenates or particle-free extracts of rat liver *Arch. Biochem.* 43, *231 (1953)*
11. VAN BAALEN, J, and GURIN, S. Cofactor requirements for lipogenesis. *J. Biol. Chem* 205, *303 (1953)*
12 BLOCH, K : The biological synthesis of lipids *Cold Spring Harbor Symp. Quant. Biol* 13, *29 (1948)*.
13 DRURY, D R · Role of insulin in carbohydrate metabolism *Am J Physiol* 131, *536 (1940)*.
14 STETTEN, DEW, JR, and BOYER, G E.: Studies in carbohydrate metabolism III Metabolic defects in alloxan diabetes *J Biol Chem* 156, *271 (1944)*.
15 BRADY, R O, and GURIN S : Biosynthesis of labeled fatty acids and cholesterol in experimental diabetes. *J Biol Chem* 187, *589 (1950)*
16 BAKER, N, CHAIKOFF, I L., and SCHUSDEK, A · Effect of *fructose on lipogenesis from lactate and acetate in diabetic liver J Biol Chem* 194, *435 (1952)*.
17 LYON, I, MASRI, M S, and CHAIKOFF, I L Fasting and hepatic lipogenesis from C^{14} acetate *J Biol. Chem* 196, 25 *(1952)*
18 VAN BRUGGEN, J T, HUTCHENS, T. T, CLAYCOMB, C K, CATHEY, W. J., and WEST, E S. The effect of fasting upon *lipogenesis in the intact rat J Biol Chem 196, 389 (1952)*
19 MASORO, E J, CHAIKOFF, I L, CHERNICK, S S, and FELTS, J M Previous nutritional state and glucose conversion to fatty acids in liver slices *J Biol Chem* 185, *845 (1950)*
20 RABINOWITZ, J L., and GURIN, S Biosynthesis of cholesterol and β-hydroxy-β-methylglutaric acid by extracts of liver *J Biol. Chem* 208, *307 (1954)*
21 BUCHER, N L R The formation of radioactive cholesterol and fatty acids from C^{14}-labeled acetate by rat-liver homogenates *J Am Chem Soc* 75, *498 (1953)*
22 SHORR, E The relation of hormones to carbohydrate metabolism *in vitro Cold Spring Harbor Symp Quant. Biol* 7, *323 (1939)*
23 ARTOM, C Role of choline in the oxidation of fatty acids by the liver *J Biol Chem* 205, *101 (1953)*
24 LOEBEL, R O Beitrage zur Atmung und Glykolyse tierischer Gewebe *Biochem Ztschr* 161, *219 (1925)*.
25 WELIN, G Needle biopsy and liver function tests in acute hepa *titis and cirrhosis of the liver Acta med. scandinav* 143, *Suppl 268 (1952)*
26 AMATUZIO, D S, and NESBITT, S. Study of pyruvic acid in blood and spinal fluid of patients with liver disease with and without hepatic coma *J Clin Investigation 29, 1486 (1950)*

27. BRADY, R O, LUKENS, F. D W, and GURIN, S: Synthesis of radioactive fatty acids in vitro and its hormonal control *J Biol. Chem* 193, *459 (1951)*
28. MEDES, G, THOMAS, A, and WEINHOUSE, S Nutritional factors in fatty acid synthesis by tissue slices in vitro *J Biol Chem* 197, *181 (1952)*.
29. LYNEN, F., REICHERT, E, and RUEFF, L. Zum biologischen Abbau der Essigsäure VI "Aktivierte Essigsaure", ihre Isolierung aus Hefe und ihre chemische Natur *Justus Liebig's Ann Chem* 574, *1 (1951)*
30. ROSSLE, R Entzundungen der Leber *Handbuch der Speciellen Pathologischen Anatomie und Histologie* F Henke and O Lubarsch, Editors Vol V, part 1, Berlin, Springer, 1930 (p 250)
31. POPPER, H Distribution of vitamin A in tissue as visualized by fluorescence microscopy *Physiol. Rev* 24, *205 (1944)*
32. CHIPPS, H D, and DUFF, G. L. Glycogen infiltration of liver cell nuclei *Am J Path* 18, *645 (1942)*.
33. ZIMMERMAN, H J, MACMURRAY, F G, RAPPAPORT, H, and ALPERT, L K. Studies of the liver in diabetes mellitus, structural and functional abnormalities *J Lab & Clin Med* 36, *912 (1950)*
34. DAVIDSON, J D Diet and lipotropic agents in arteriosclerosis *Am J Med* 11, *736 (1951)*

CARDIOVASCULAR LESIONS IN CHOLINE-DEFICIENT RATS*

W. STANLEY HARTROFT
Banting and Best Department of Medical Research
University of Toronto

THE EXPERIMENTS I shall speak of had their origin in studies on the liver in choline deficiency; I do not think they have any clinical significance at the present time. Seven years ago, Kesten and Salcedo, working with Dr. Stetten at Columbia (1) reported that young rats fed a low protein diet, containing 35 to 40 per cent of the ethyl ester of the short-chain fatty acid, lauric acid, died within 3 to 6 days of a massive cardiac necrosis These heart lesions could be prevented by supplements of choline added to the diet

Two years ago we attempted to confirm this observation, but our rats failed to consume the food mixture containing ethyl laurate, and died from inadequate caloric intake without, of course, developing any cardiac lesions. It was not until a year ago that a newcomer to our department, Dr George F. Wilgram, at Dr. Best's suggestion, took up the problem By perseverance, he succeeded in confirming Dr Stetten's observation concerning the cardionecrogenic action of ethyl laurate in low choline diets, although his rats did not die as rapidly, nor was the cardiac necrosis as extensive as reported by the Columbia investigators.

In our rats, fed ethyl laurate in a choline-deficient food mixture, cardiac necrosis was preceded and accompanied by the deposition of abundant amounts of stainable fat in the muscle fibers of affected hearts Kesten, Salcedo, and Stetten, had looked for similar fat deposits in hearts of their animals and reported their absence We think this apparent discrepancy may be explained by a consideration of the stage of cardiac necrosis involved. In the earlier stages of the lesion, stainable fat in affected muscle cells is abundant, (Figure 14), later when the cells have undergone necrosis and lysis the fat escapes and is removed by ophages Examination of necrotic hearts at this stage r i fat, for it has largely been removed. The fore very well

* This work was carried out in collabor ... am, of the Banting and Best D... ment of Medica ... o

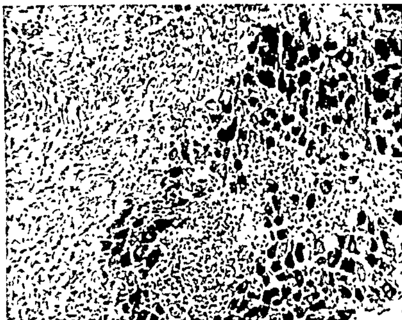


FIGURE 14 Frozen section, stained with Oil red O to demonstrate fat, prepared from the heart of a choline-deficient rat This field illustrates several stages in the production of the type of cardiac damage described in the text At the periphery of the lesion, cardiac muscle cells are filled with stainable fat deposits (black in photograph) In the more advanced portions of the lesion, muscle cells have become lysed with release of fat droplets, which have been removed by macrophages X300

account for the differences between the findings of Kesten, Salcedo, and Stetten, and our own, with regard to stainable fat.

At Toronto, we then investigated the effect of choline-deficient diets which contained synthetic triglycerides other than lauric acid or its ethyl ester Tricaproin, tricaprylin, trimyristin and tripalmitin, when incorporated into choline-deficient diets at levels of 25 to 35 per cent, were all effective in producing varying degrees of cardiac necrosis in young male rats consuming these food mixtures. When all fat was omitted from the basal choline-deficient ration and replaced by sucrose, a small percentage (20 per cent) of the rats fed this diet also developed lesions in their hearts Cardiac necrosis and more than traces of stainable fat in heart muscle were absent in rats fed any of these experimental diets supplemented with choline chloride (0.85 per cent) We concluded, then, that the

essential cause of this type of cardiac damage was dietary choline deficiency *per se*, and that this could be intensified by the addition of a high percentage of synthetic triglyceride to the diet Compounds of lauric acid were more effective in increasing the incidence of the lesion than were other triglycerides tested, and to this extent our observations were in agreement with those of Kesten, Salcedo and Stetten They had concluded that only derivatives of lauric acid were cardionecrogenic in choline-deficient diets Our experiments indicated, however, that the essential condition for production of this lesion was dietary choline deficiency, and that ethyl laurate, instead of being necessary for its production, intensified the effect of choline deficiency and rendered its effect on the heart more dramatic and easily demonstrable

These results prompted a series of experiments in which naturally occurring fats (beef, fat, lard, corn oil, and coconut oil) were fed at comparably high levels (25 to 35 per cent) in this type of choline-deficient diet. Rats consuming these food mixtures developed varying degrees of lipocardiosis (fat in heart muscle) and cardionecrosis. Beef fat and lard, under these conditions, were associated with a higher incidence of the lesions than were corn oil or coconut oil. Over 90 per cent of young male rats (100-120 gm), which were fed a choline-deficient diet containing 35 per cent lard, and 30 per cent methionine-poor protein, developed cardiac lesions These changes in the hearts of the animals were frequently, but not necessarily always, associated with the development of acute renal lesions It appears at the present time that kidney damage may intensify the effect of choline-deficient diets on heart muscle, but is not an essential factor for this result

Recently we have observed both gross and microscopic lesions in the aortas and coronary arteries of young male rats consuming choline-deficient diets of this type (Figure 15). Deposits of stainable fat have been observed in the walls of coronary arteries, and in the aortas both fatty and calcium deposits have been found in intima and media respectively. Grossly sclerotic lesions in the aorta have been observed within periods as brief as 17 days in young male rats fed on these diets. The changes appear to be the acute version of the ones observed in older rats fed less severe hypolipotropic diets containing smaller percentages of fat, over periods of 4 to 6 months (2)

The deposition of stainable fat in hearts and arteries as well as in kidneys and livers of choline-deficient rats suggests that the lipotropic action of choline and its dietary precursors is not limited

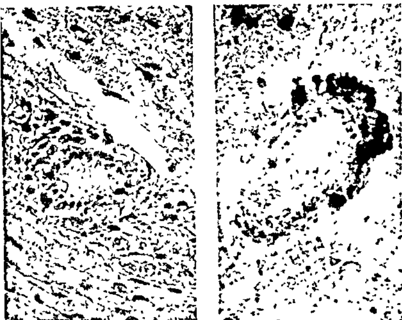


FIGURE 15 On the left is a normal arterial branch in the heart of a choline-supplemented control rat On the right is a similar preparation from a choline-deficient animal Both sections have been stained with Oil red O to demonstrate stainable fat The wall of the artery in the choline-deficient rat contains stainable fat, chiefly in the media, with a small amount in the intima X300

to the liver alone The concept appears to be emerging from these and other investigations that adequate amounts of dietary choline are necessary, not only for the maintenance of a normal and healthy liver, but also for the preservation of a normal cardiovascular-renal system This approach is perhaps further supported by the finding, previously reported from our laboratories, that brief periods of choline deficiency in the early life of young rats may be associated with the development of hypertension later (3)

Goldblatt Dr Hartroft, although you did not mention anything about the elastica in the media, I am sure you must have studied it. I am thinking about the work of Blumenthal, Lansing, and others (4,5) on the primary degenerative changes in the media

Hartroft Microscopic changes in the *elastica lamina* do not appear to constitute a primary feature of the lesion We have not employed microincineration methods, but histochemical techniques for the demonstration of stainable calcium deposits have failed to

reveal any abnormal calcification in the early stages of the lesion, encountered either in the aortas, or coronary arteries, of affected rats. Stainable fat deposits in the arterial walls appear to precede quite clearly any later calcification in the aorta. In the coronary arteries we have not yet encountered abnormal calcification, and have only observed deposits of mural lipid.

Necfe: In the acute infarct in the human, does one see this fat deposition in the muscle cell?

Hartroft: I believe stainable fat deposits in cardiac muscle are frequently encountered in acute infarcts of the heart in man

Popper There is a stage where it can be seen

Hartroft. Both in the case of cardiac infarcts in man, and in the type of cardiac necrosis encountered in the choline-deficient rat, the amount of stainable fat deposits observed depends on the stage of the lesion observed. Before cellular lysis has occurred in the affected regions, the muscle cells frequently contain abundant amounts of fat. With further advance of the necrosis and dissolution of cell membranes, the fat is liberated into tissue spaces where it is engulfed by macrophages and removed Thus the more advanced the necrosis, the less the amount of fat in muscle fibers, and conversely.

Best. The rat is a difficult animal in which to produce lesions of this type. In these experiments there has been no added cholesterol, therefore, one of the next steps will be to add it. Dr. Hartroft is carrying this out at the present time.

Lillie This process is decidedly reminiscent of dietary heart lesions which Ashburn showed me in his laboratory about ten years ago He and Daft (6) and some others tried to repeat the experiment, and it did not happen again.

Best Was that a low-choline diet?

Gyorgy The usual low-choline diet

Madden: I think Wissler (7) reported the production of this lesion in rats on a lard diet I do not believe he specified that it was low choline, and I do not know whether this was measured High choline intake was noted in later report (8).

Hartroft: The animals which developed acute lipomatous and atheromatous lesions (9) of coronary arteries were force-fed a high-fat, high-choline ration containing cholesterol, and with one per cent NaCl in their drinking water were subjected to anti-rat-kidney serum, DCA, and sesame oil injections. In chronic experiments lasting a year, lipomatous lesions of the coronary arteries were observed in a group receiving high levels of lard, high-choline supplements,

and added cholesterol. I do not know whether Wissler observed gross lesions in the animals' aortas The presence of the lesions in rats, given choline supplements, is difficult to correlate with our findings. We have never encountered vascular abnormalities in large numbers of control rats fed our basal diets supplemented with 0 85 per cent choline chloride Experiments in our laboratory, currently underway, include rats being fed our basal diet with added cholesterol, in the presence and absence of choline supplements.

Madden: What is the degree of choline deficiency?

Hartroft: It is very severe. The high levels (25 to 35 per cent) of fat are one factor, and the high level of methionine-poor protein (30 per cent) is another The diet which we have employed most extensively in these experiments contain 30 per cent alcohol-extracted peanut meal, 5 per cent alpha soya protein, and 10 per cent casein. These diets are then high in fat, high in protein and *low in choline*

Madden. And low in methionine?

Hartroft: Yes, although the protein content is 30 per cent, the diets contain only 440 to 530 mg of methionine per 100 grams of food mixture

Madden: Are they vitamin-supplemented?

Hartroft. Yes, the diets contain a vitamin supplement which we routinely employ in most of our dietary experiments (10).

Best: Some day we may be able to write a paper on the production, in 17 days, and by dietary deficiency, of the signs of advanced old age in young rats, but we have quite a long way to go before that will be possible

Hartroft Of the natural fats studied by incorporating them at levels of 25 to 35 per cent in this type of basal diet, coconut oil did not result in a very high percentage of cardiac lesions in rats consuming the food mixture This was somewhat surprising as coconut oil contains a large amount of lauric acid

Best This cardiac necrosis occurs in 100 per cent of these experiments, but it is absent with choline

Hartroft Young rats consuming the high fat (35 per cent lard), high protein, and low-choline diets, developed cardiac lesions in *almost every instance, controls fed the same diet with choline* added were free of significant changes in their hearts.

Popper I assume you did not find any mural thrombi in the heart muscle

Hartroft At the time of autopsy, no attempt was made to remove blood or blood clot from the chambers of the heart in order to pre-

reveal any abnormal calcification in the early stages of the lesion, encountered either in the aortas, or coronary arteries, of affected rats. Stainable fat deposits in the arterial walls appear to precede quite clearly any later calcification in the aorta. In the coronary arteries we have not yet encountered abnormal calcification, and have only observed deposits of mural lipid.

Neefe: In the acute infarct in the human, does one see this fat deposition in the muscle cell?

Hartroft: I believe stainable fat deposits in cardiac muscle are frequently encountered in acute infarcts of the heart in man

Popper: There is a stage where it can be seen.

Hartroft. Both in the case of cardiac infarcts in man, and in the type of cardiac necrosis encountered in the choline-deficient rat, the amount of stainable fat deposits observed depends on the stage of the lesion observed. Before cellular lysis has occurred in the affected regions, the muscle cells frequently contain abundant amounts of fat. With further advance of the necrosis and dissolution of cell membranes, the fat is liberated into tissue spaces where it is engulfed by macrophages and removed. Thus the more advanced the necrosis, the less the amount of fat in muscle fibers, and conversely.

Best: The rat is a difficult animal in which to produce lesions of this type In these experiments there has been no added cholesterol, therefore, one of the next steps will be to add it Dr. Hartroft is carrying this out at the present time

Lillie: This process is decidedly reminiscent of dietary heart lesions which Ashburn showed me in his laboratory about ten years ago He and Daft (6) and some others tried to repeat the experiment, and it did not happen again

Best Was that a low-choline diet?

Gyorgy: The usual low-choline diet

Madden. I think Wissler (7) reported the production of this lesion in rats on a lard diet. I do not believe he specified that it was low choline, and I do not know whether this was measured High choline intake was noted in later report (8)

Hartroft The animals which developed acute lipomatous and atheromatous lesions (9) of coronary arteries were force-fed a high-fat, high-choline ration containing cholesterol, and with one per cent NaCl in their drinking water were subjected to anti-rat-kidney serum, DCA and sesame oil injections In chronic experiments lasting a year, lipomatous lesions of the coronary arteries were observed in a group receiving high levels of lard, high-choline supplements,

and added cholesterol I do not know whether Wissler observed gross lesions in the animals' aortas. The presence of the lesions in rats, given choline supplements, is difficult to correlate with our findings We have never encountered vascular abnormalities in large numbers of control rats fed our basal diets supplemented with 0 85 per cent choline chloride. Experiments in our laboratory, currently underway, include rats being fed our basal diet with added cholesterol, in the presence and absence of choline supplements

Madden: What is the degree of choline deficiency?

Hartroft: It is very severe. The high levels (25 to 35 per cent) of fat are one factor, and the high level of methionine-poor protein (30 per cent) is another The diet which we have employed most extensively in these experiments contain 30 per cent alcohol-extracted peanut meal, 5 per cent alpha soya protein, and 10 per cent casein These diets are then high in fat, high in protein and low in choline.

Madden. And low in methionine?

Hartroft Yes, although the protein content is 30 per cent, the diets contain only 440 to 530 mg of methionine per 100 grams of food mixture

Madden· Are they vitamin-supplemented?

Hartroft: Yes, the diets contain a vitamin supplement which we routinely employ in most of our dietary experiments (10)

Best Some day we may be able to write a paper on the production, in 17 days, and by dietary deficiency, of the signs of advanced old age in young rats, but we have quite a long way to go before that will be possible.

Hartroft. Of the natural fats studied by incorporating them at levels of 25 to 35 per cent in this type of basal diet, coconut oil did not result in a very high percentage of cardiac lesions in rats consuming the food mixture Thus was somewhat surprising as coconut oil contains a large amount of lauric acid

Best This cardiac necrosis occurs in 100 per cent of these experiments, but it is absent with choline

Hartroft Young rats consuming the high fat (35 per cent lard), high protein, and low-choline diets, developed cardiac lesions in almost every instance, controls fed the same diet with choline added were free of significant changes in their hearts

Popper I assume you did not find any mural thrombi in the heart muscle

Hartroft At the time of autopsy, no attempt was made to remove blood or blood clot from the chambers of the heart in order to pre-

Popper: No, I meant arteriosclerotic vascular changes.

Hartroft: We have not observed vascular lesions in the renal arteries.

Madden: Have you tried it in adult animals?

Hartroft: Yes; Ridout, Sellers, Best, and I (2) observed this type of vascular damage in choline-deficient diets for the first time in young, adult, male rats, and reported these findings a little more than a year ago. I referred to these changes earlier as chronic lesions. The animals in which they were observed weighed 180-200 gm. at the beginning of the experiment. They were maintained on a low-choline diet for periods ranging from 4 to 6 months The total age of the animals at the time of sacrifice was still under 300 days, however. The aortas of many of these rats were grossly sclerotic, and in the coronary arteries of many of their hearts, fat deposits were found in intima and media. There was intimal hyperplasia in many of the vessels of the heart The fat content of the diet fed these older rats was less than in the acute experiments we have reported today, and contained 20 per cent hydrogenated vegetable fat, called "Primex."

Popper. Do you assume that the alteration of the myocardial fibers is the result of anoxia due to obstruction of small vessels, or do you think it is independent from the vascular lesions?

Hartroft: I cannot give a definite answer to that question. Frequently the vascular and parenchymal changes in the heart appear to progress hand-in-hand. In some instances, however, we have encountered lesions in vessels without changes in heart muscle, and conversely.

Madden· May I ask if you have tried to reverse the lesions?

Hartroft: No. That is a good experiment which will have to be done.

Bollman. What changes are there in the blood?

Hartroft Dr Jessie H Ridout, in our laboratory at the Banting and Best Department of Medical Research, studied the total serum lipids, and the serum cholesterol, in the choline-deficient rats with vascular lesions. She found that the level of serum cholesterol in the rats with lesions was significantly less than in the cholinesupplemented controls.

REFERENCES

1. KESTIN, H D, SALCEDO, J, JR, and STETTEN, DEW, JR Fatal myocarditis in choline deficient rats fed ethyl laurate *J Nutrition 29, 171 (1945)*

2. HARTROFT, W S, RIDOUT, J H, SELLERS, E A, and BEST, C. H Atheromatous changes in aorta, carotid and coronary arteries of choline-deficient rats *Proc. Soc Exper. Biol. & Med* 81, *384 (1952).*
3 HARTROFT, W. S, and BEST, C H · Hypertension of renal origin in rats following less than one week of choline deficiency in early life *Brit M J* 1, *423 (1949)*
4 BLUMENTHAL, H T, LANSING, A. I, and GRAY, S H The interrelation of elastic tissue and calcium in the genesis of arteriosclerosis *Am J Path* 26, *989 (1950)*
5 LANSING, A I Some physiological aspects of ageing *Physiol Rev* 31, *274 (1951)*
6 DAFT, F. S, ASHBURN, L L, SPICER, S S, and SEBRELL, W H. The occurrence of hyaline sclerosis and calcification of blood vessels in rats on sulfaguanidine *Public Health Rep* 57, *217 (1942)*
7 WISSLER, R W, EILERT, M L, SCHROEDER, M A, and COHEN, L Arterial atheromatous lesions in rat *Federation Proc.* 11, *434 (1952)*
8 WISSLER, R W, COLLINS, J L, SCHROEDER, M, and SOULES, K Factors responsible for lipomatous and atheromatous lesions in coronary arteries of rat *Federation Proc* 12, *407 (1953)*
9 WISSLER, R W The production of atheromatous lesions in the albino rat *Proc Inst Med Chicago* 19, *79 (1952)*
10 BEST, C H, LUCAS, C C, PATTERSON, J M, and RIDOUT, J H Some effects of vitamin B_{12} in weanling rats consuming hypolipotropic diets *Canad J Med Sc* 31, *135 (1953)*
11 DAVIES, J N P Kwashiorkor *Liver Injury* F W Hoffbauer, Editor Trans Ninth Conf New York, Josiah Macy, Jr Foundation, 1951 (p 151)
12 WEISS, S, and WILKINS, R W The nature of the cardiovascular disturbances in the nutritional deficiency states (beriberi) *Ann Int Med* 11, *104 (1937)*
13 DOCK, W Marked cardiac hypertrophy and mural thrombosis in the ventricles in beriberi heart *Tr A Am Physicians* 55, *61 (1940)*
14 SMITH, J J, and FURTH, J Fibrosis of endocardium and myocardium with mural thrombosis notes on its relation to isolated (Fiedler's) myocarditis and to beriberi heart *Arch Int Med* 71, *602 (1943)*
15 NELSON, D, IVY, A C, SZANTO, P B, and POPPER, H Generalized metastatic calcification combined with vascular changes produced by diet *Federation Proc* 9, *339 (1950)*

THE LIVER AND PROTEIN METABOLISM*

H. W. KOSTERLITZ
Department of Physiology
University of Aberdeen
Aberdeen, Scotland

WE MAY APPROACH the relationship between protein metabolism and the liver in two ways; either by considering the effect of the liver on protein metabolism, or in reverse, the effect of protein metabolism on the composition and structure of the liver. These relationships exist also as far as carbohydrate and fat metabolism are concerned, but in the case of protein metabolism, the changes which we observe in the liver are much more pronounced and apparently have greater importance.

I intend to subdivide my presentation into roughly three parts: In the first section, I shall deal with the problem of a standard of reference for the analyses of liver tissue. In the second, I shall consider the effects of dietary proteins on the composition of the liver, and in the last section, discuss the effects of some hormones on the constituents of liver tissue, with particular reference to ribonucleic acid (RNA).

So as far as a standard of reference for liver analyses is concerned, this is an old and rather difficult problem. If we wish to express results of glycogen and triglyceride analyses, then we may overcome the difficulty which is caused by the rapid changes in the weight of the liver by referring the results to fat-free and glycogen-free dry liver tissue. However, if there are simultaneous changes in the protein content of the liver, this method is no longer applicable. These changes may occur very rapidly, within a few hours, and may amount to as much as 25 per cent and more of the protein content of the liver. The obvious conclusion would be to express the results in terms of units of liver cells, and to be even more definite, in terms of units of parenchymal cells or hepatocytes. As a measure of the number of cells present, we could take the nuclei, and as a measure of the nuclei, the content of desoxyribonucleic

* Most of the results reported in this paper were obtained in collaboration with Mrs Rosa M Campbell The work was generously supported by the Medical Research Council of Great Britain

acid (DNA). The difficulty which arises is that there are other cells

relative change between hepatocytes and nonhepatocytes in regenerative processes, and, of course, in cirrhosis. However, for short-term experiments, the type which I wish to describe here, there is no evidence of a significant change in the relationship between hepatocytes and other cells

The constancy of the DNA content of nuclei was first shown by

en shown that at
the liver nuclei
as, for instance,
keeping a rat on a protein-free diet for four weeks (2). A very detailed examination of this problem of whether dietary changes affect the average DNA content of liver nuclei has recently been published from the laboratory of J N. Davidson in Glasgow (3). He and his collaborators demonstrated that neither age, sex, nor diet had any effect. This may be rather surprising, because we know that particularly in the livers of rats and other rodents, polyploidy is very marked

Figure 16 is taken from the lecture Dr Davidson gave last year to the International Biochemical Congress (4). The amount of DNA in the nuclei was determined photometrically by means of the Feulgen reaction. In the kidney we have only one class of nuclei, but in the liver we have, apart from the diploid nuclei, a large number of tetraploid nuclei, and a small number of octaploid nuclei *A priori*, it is by no means certain that changes in diet will not affect the relative numbers of diploid, tetraploid and octaploid nuclei, but from all the results which are available so far, whether the average DNA content of isolated nuclei, or the DNA content of the whole liver is estimated, there is no evidence of any change in polyploidy during short-term experiments. Thus, there seems to be a good case for using liver DNA as a standard of reference

One difficulty which I should like to point out in this connection, is of a rather technical nature The degree of homogenization of the liver in the process of isolating the nuclei, determines how many nuclei other than hepatocytes will appear in the suspension, because *the nuclei of hepatocytes are most readily dislodged from* their cells Finally, I should mention that the results to be reported later do not distinguish between intracellular and extracellular constituents

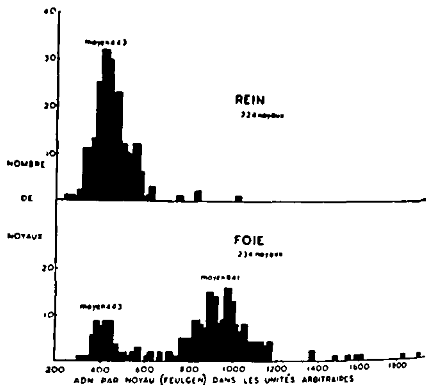


FIGURE 16 The DNA content of isolated nuclei of the kidney and liver of the rat, determined photometrically after staining by the Feulgen reaction Reprinted by permission, from Davidson, J N Les nucléoproteines et la croissance des tissus *Bull Soc chim biol* 35, 49 (1953)

The DNA content of the whole liver is determined solely by the size and sex of the animal (5) Figure 17 shows the amount of DNA (mg) in the whole liver of the different animals, both abscissa and ordinate being on the logarithmic scale. We see that at least for the range from mice to guinea pigs, the relationship between log DNA and log body weight, is linear.

Figure 18 shows the DNA content of rat livers of different weights. In the lower part of the curve we have young immature rats, then there is a gap for which I have no data, and in the upper part are adult mature rats. The open circles indicate the DNA content of the livers of female rats, and the dots that of male rats It would appear from this graph that, for a given body weight, the female rat has more DNA in the liver than the male Since there is no sex difference in the average DNA content of the adult nuclei,

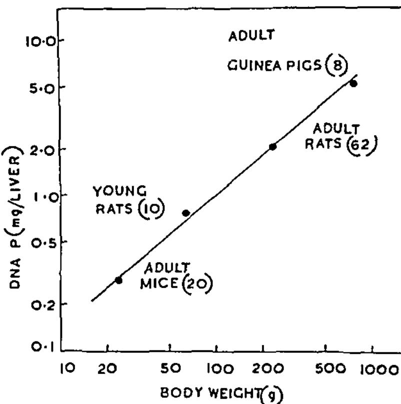


FIGURE 17 The relationship between log DNA P and log body weight for female mice, rats and guinea-pigs The line was drawn from the regression equation log DNA P (mg) = 0 845 × log body weight (gm) − 1 67 Figures in brackets indicate number of observations

it also means that for a given body weight the female rat has more liver cells than the male

We have demonstrated that the DNA content of the liver is independent of changes in the dietary protein, fat, energy, and choline contents I shall not show you the evidence for that, because it is, of course, negative (2,5,6) However, Figure 19 will, I hope, help you to appreciate the advantage which can be derived from referring the results to DNA

The first four columns represent the protein nitrogen content expressed in mg per 100 gm of liver The first column is that of

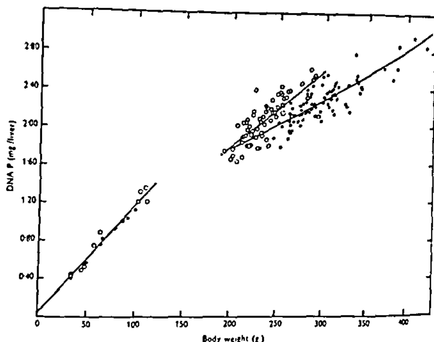


FIGURE 18 The relationship between the DNA content of the liver, and the body weight. The dots indicate male, and the circles female rats. The lines were drawn from regression equations Reprinted, by permission, from Campbell, R M and Kosterlitz, H. W.: The effects of growth and sex on the composition of the liver cells of the rat. *J Endocrinol. 6, 308 (1950).*

a normally fed animal, and the concentration is approximately 2 8 per cent. The second column is that of a rat which had been fed on a protein-free diet, and the concentration is down to 1.9 per cent. The third column is that of a fasted animal and the concentration is about the same as in the normal animal. The last column shows the high protein concentration in the liver of an animal which had been hypophysectomized, and in consequence of that operation, had reduced its food intake.

If we now determine the concentration of DNA in these livers, we find similar values in the livers of the rats fed on adequate or protein-free diets In the fasted rat the concentration of DNA has gone up, because of the loss of glycogen and water from the liver. This increase in the DNA concentration is still more marked in the hypophysectomized animal. The total DNA present in these livers is about the same, provided there are no differences in body weight.

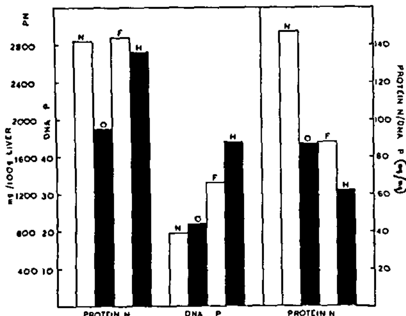


FIGURE 19 The prot... (N) or a protein-fr... sectomized rats fed per 100 gm liver group of columns) per 100 gm liver

If we now express the results in terms of mg. protein N per mg DNA P, we have a true picture of what is happening. The first column represents the protein content of a given number of liver cells After keeping the animal on a protein-free diet for, say, a week, the value is reduced In the fasted animal a similar amount of protein has been lost, although the concentration is as high as in the normal rat In the hypophysectomized animal the protein content of the liver cells has fallen even more than in the fasted rat.

Thus, I believe there is a great advantage in expressing the analytical results in this way However, caution has to be exercised, because there may be changes in the average DNA content of the liver cells This may be caused by a variation in polyploidy, or by an increase in the number of cells which are not hepatocytes. Furthermore, there may be an abnormal accumulation of DNA in the nuclei, as was shown, for instance, by Ely and Ross (7), in

experiments in which young rats were kept on a protein-free diet for periods of from three to four weeks.

An alternative method would be to express the results in terms of the whole liver; or if the animals were not of the same body weight, per 100 gm. of body weight. However, this would be only a second best way of proceeding

Best: I think we might have some discussion on the problem of a standard of reference for the analysis of liver tissue at this point.

Vars: Would a short experiment in connection with the variations in polyploidy be limited to two days, one day, or eight hours?

Kosterlitz: No, much longer than that. In animals fed on a protein-free diet, we may safely continue for three or four weeks I have not seen evidence that there is a variation in polyploidy While we have not actually determined the relative number of diploid and tetraploid nuclei, there is no positive evidence that such variation occurs There is no change in the average DNA content of the liver nuclei, and there is no change in the total DNA content of the liver.

Popper: I am sure some of you will remember that the same question was brought up in these conferences several years ago, when changes in nucleic acid content and concentration in the liver were reported as resulting from carbon tetrachloride and ethionine intoxication (8). From the discussion in which Drs. E S Gordon, Philip Handler, Harold Tarver and DeWitt Stetten participated, the conclusion was reached that the most suitable method was to take as reference point the body weight of the rats at the beginning of the experiment, in order to avoid the question of polyploidy, changes in DNA and what not. I should like to ask which reference point would appear suitable in experiments which are too protracted to use the initial body weight, because of growth of the rats or the like. The body weight changes also in short-term experiments, but this change will not interfere with the interpretation, if one takes as reference point for the concentration of a substance in the liver the initial body weight at the beginning of the experiments and ignores any changes of the body weight resulting from fasting (often for two or three days) or other experimental factors

Kosterlitz I think all the circumstances have to be taken into consideration If we have a simple nutritional experiment in which we can assume there is no change in the number of liver cells, then I think expressing the results per 100 gm. of initial body weight is all right However, if we have a situation as in pregnancy, where

the number of liver cells is increased, then to express the results per 100 gm. of body weight would be misleading. We may wrongly conclude, from increased protein values, that there is an increase in the protein content of the liver cell, although the results may be accounted for by the increased number of cells. We find the same situation in carbon tetrachloride poisoning There is an increase in the protein content, but that is due mainly to the formation of new liver cells

Popper In carbon tetrachloride poisoning, there are changes in the nuclei and tissue which seem to invalidate DNA as a reference point You mentioned loss of cells, and the appearance of scavenger cells which are not liver cells That may disturb us if we wish to know whether there is more protein in the liver cells, or whether it is distributed in more or fewer cells. All of us who make analyses of liver tissue are confronted with this problem, and are in urgent need of a suitable reference point

Kosterlitz: In carbon tetrachloride poisoning, I personally would be very hesitant to do any analysis of this type after twenty-four hours, because up to that time, if there had been an invasion of foreign cells, it would be very slight; that is, expressed in the percentage of the number of hepatocytes. Would you not agree with that?

Popper. I do not know.

Kosterlitz: We looked at these livers and found very few foreign cells When there is necrosis of cells in carbon tetrachloride poisoning, we do not know whether DNA is lost or can no longer be demonstrated by histological staining methods. From the chemical analyses that we have made, we rather feel that it is still there

Popper It would be advantageous if all of those who are doing analyses in liver tissue would agree on one reference point in order to permit comparison of the findings in different laboratories So far, a really suitable reference point does not seem to exist, and using the initial body weight of the animals is probably the most primitive of any method

Kosterlitz Under these circumstances, if we wish to obtain a full analysis, we have to analyze the liver, and know the liver and body weight Furthermore, we have to count the number of nuclei We have to determine the degree of polyploidy, and also estimate the average DNA content of the nuclei No one has done that so far, and I think we cannot answer the questions you have put until this problem has been fully investigated

Best If you were determining desoxyribonucleic acid, what would you use as a standard of reference?

Kosterlitz: We use the body weight of the animal. I showed you the regression lines. In Figure 18, the regression line is very constant for a given strain of rats; we have redetermined it at intervals of one or two years, and the numerical values of the equation vary only a little. That is to say, there is no significant difference in the regression coefficient, although there may be slight variation in the absolute terms (4,6). Thus, when we determine the DNA content of a liver, we can say whether it is greater or smaller than that which we would expect for a normal rat in the colony.

Best: What I was trying to bring out was that there could be no single standard of reference. We must have more than one

Kosterlitz: I quite agree with you that there cannot be just one Strictly speaking, when we say a substance has been determined in the liver of a rat, that is the first standard of reference giving the species; then comes the sex, age, type of strain, and so on. However, so far as we know, in a normal rat the DNA content of the liver is determined only by the body weight and sex.

Gurin: I think Dr. Aaron Bendich (9) found recently that DNA fractions are mixtures of several DNA's. Wouldn't that introduce another possibility of variability in this analytical method? I have no idea how the proportions or the phosphorus components would vary in the individual DNA components.

Kosterlitz: As far as the liver of the adult rat is concerned, we are in no difficulty, because it contains only one type of DNA In other organs, there are two types.

Best: Do they give the same color reaction?

Gurin: I would expect them to. I wonder how the proportion would change under various experimental conditions I do not suppose there is any information on that.

Kosterlitz: There is no information whatsoever. All one can say is that at present there is no direct evidence of any such variations because of the consistency of the results obtained so far. However, I quite agree that one ought to look out for such evidence

Popper: The problem is obviously even more complicated in the human If we wish to make analyses of liver biopsy specimens, body weight, or some similar figure, is obviously not suitable, and DNA probably represents the best reference point, as you suggest in your papers My question should not be interpreted as an argument against your statement; I just wished to take advantage of the present group to obtain some suggestions as to what reference point to use As a morphologist, I am still a little hesitant about accepting something which changes as much, in the morphologic picture, as

does the nuclear chromatin which reflects DNA. I do not mean to imply that our visual impression may not mislead us. We found also that apparent alterations in DNA concentration were nullified if the body weight was taken into consideration. Moreover, DNA determinations are not the most pleasant to perform.

Watson: Do your values of DNA depend upon homogenization and measurement of the optical density of the Feulgen solution, or on sections? I do not understand how you obtained those values

Kosterlitz: I am sorry I did not mention that. No, these are all chemical analyses. The DNA is determined chemically in relatively large samples: usually 2 gm of liver

Watson Does that depend on the Feulgen solution in the last analysis?

Kosterlitz: No, it is a Schmidt and Thannhauser analysis. As far as Dr. Popper's question is concerned, I think he is posing an insuperable problem. In biopsy samples, the error of sampling becomes the preponderant difficulty. In this work we use 2 gm of liver, which is a large percentage a rat's liver. We have also used the same method when we could not take out the whole liver, in cat experiments, and it still worked. However, when one tries to do that on a few milligrams of a human liver, an organ which weighs several kilograms, I think the error of sampling cannot be overcome.

Popper. I wish to support Dr. Kosterlitz by reporting that Dr. Robert Shank, in St Louis, in his studies on human liver biopsy specimen, uses DNA as a reference point. I only brought up the question so that if several of us should do lipid or other analyses in human liver tissue, we would agree on the best reference point.

Best. How much data are there from a comparative study of the histological and chemical procedures, for identifying this compound?

Popper As far as fat (10), glycogen (11), and vitamin A (12), are concerned, the correlation seems to be good However, iron is poor (13) These are the four substances which I can remember now, I am sure some of you know of other examples.

Vars In the case of biopsy material, irrespective of the analytical possibilities of the sample obtained, there is the known fact that we can analyze only a small fraction of the whole, and there is no assurance that it represents a true metabolic pattern of what is taking place in the organ as a whole I think we should proceed by histologic study, by an estimate of how much connective tissue there is and how many parenchymatous cells, and by considering other units of reference

Shorr: Have you ever tried the Latapie mincer, instead of the homogenizer, to separate the hepatocytes?

Kosterlitz: No, I have not.

Shorr: I recall one such effort on my part a number of years ago It is my recollection that with the Latapie mincer, and gentle differential centrifugation, it was possible to separate the hepatocytes from other portions of the liver tissue.

Kosterlitz: I should say that once we have established the fact that the average DNA content of the nuclei under the conditions of the experiment remains constant, we do not need to isolate the nuclei any longer. All we have to do is to determine DNA in the sample. However, we have to satisfy ourselves first that the average DNA content of the nuclei does not change. We know it does not in short-term dietary experiments.

Popper: Even in regeneration, DNA changes surprisingly little when objective measurements of individual cells are made, if I quote Dr. Vars correctly.

Kosterlitz: I think in their recent paper, Thomson, Heagy, Hutchison, and Davidson (3) found that the DNA content of the nuclei of the regenerating liver is higher than normal

Vars. That is correct. It is increased, to some extent.

Popper: Stowell (14) found that in rats, following hepatectomy, the nuclear volume was markedly increased during the first 48 hours, and also the nucleic acid content was high, as I remember The period of elevation was relatively short. In mice it was apparently somewhat longer (15) Is that correct?

Vars. Yes, because the period of active mitosis does not start until around the twenty-fourth hour, and reaches a maximum, in terms of mitotic count, of from forty-eight to seventy-two hours His observations would confirm work along the same lines by Brues (16) who made counts at various stages of regeneration.

Shorr I wonder whether there is any metabolic index that one could use as a standard of reference We have always been attracted by urinary creatinine as a measure of effective tissue mass It might be interesting to attempt a correlation in a healthy standardized organism, such as the rat.

Vars In the rat, there is one enzyme that works rather well, but in the human it would not be so effective. It is the enzyme rhodanese, which seems to be of parenchymatous origin in the rat, but we know very little about its metabolic function. However, it seems to parallel protein changes in the organ. It goes its own steady way

and does not show the striking changes that other enzymes with more interesting metabolic functions do.

Hartroft: According to Bruno Mendel (17,18), rather striking differences, involving rhodanese, can be demonstrated between tumor tissue and normal tissue, using ferricyanide as a test substance.

Vars: For observations of the normal regenerative liver, it has been a good standard of reference (19).

Hartroft: Can consistent differences in the activity of rhodanese, and the DNA content, be demonstrated between tissues in stages of active hyperplasia and neoplasia? I have in mind hyperplasia such as that following partial resection of the liver. If I have understood Dr. Kosterlitz correctly, there would not be much change in DNA content of a tissue during active hyperplasia

Kosterlitz: I am sorry, I must have been confusing the issue We must differentiate between the DNA content of the whole organ, and that of the individual nucleus. In regeneration of the liver, we have a relatively small increase in the average DNA content of the nuclei, but naturally a very marked increase in that of the whole organ As a matter of fact, I do not think I am quite competent to say what the DNA content of the individual nuclei following hepatectomy is.

Popper Although I do not know of a specific measurement, circumstantial evidence indicates a slight increase but surely it is not as dramatic as one would be led to believe from the histological picture

Best Dr Watson and I were hoping we could obtain information from this group as to what people who do biopsies on human liver actually use as a standard of reference

Popper We are absolutely helpless We are using the dry weight which is, of course, the simplest procedure, but probably the least informative It at least omits edema as a source of error, but we are looking for something better I believe Schuff (10) in his study on liver lipids uses the wet weight As already mentioned, Shank, in his extensive work, uses DNA as a base line, Waterlow (20) does also

Kosterlitz If it were possible to separate the hepatocytes from all the other tissue, the DNA content would be a very good standard of reference However, I think the real trouble is that you have such a small sample

Popper There have been a few investigations along that line. Anybody who undertakes such a study will attempt to establish

the differences in various samples of the same liver, and with most of the substances these differences apparently are small, as has been shown for lipids (10,21,22), or vitamin A (12,23), at least in the normal liver. As far as glycogen is concerned, there seem to be significant differences (24). In the abnormal liver regional differences have been reported in lipids (25), for instance An histological analysis may help. The presence of marked fibrosis would have to be taken into account.

Kosterlitz: If allowance is made for the sampling error, I think the DNA content is still the best standard of reference

Lillie: I was thinking of the possibility of relating DNA content to that portion of the liver which one loses in preparing a Mall reticulum preparation. The partial digestion that Mall used to get rid of the cells so that he could demonstrate reticulum would seem to dispose of, or isolate, this stroma fraction which one could then subtract from all the rest of the sample.

Watson. That would get rid of all the nuclei, the Kupffer cells, and any leukocytes

Lillie: Certainly.

Watson: Thus, we would still be up against the same problem

Lillie: In the matter of fibrotic liver we would be able to extract the excess stroma and be closer to parenchyma. I do not think the proportion of cells would vary as much as the proportion of stroma to cells.

Watson. I was under the impression that the stroma itself had no DNA in it, anyway, and that it was only in the nuclei

Lillie. But the stroma carries stroma cells which do have DNA, and one finds it very difficult to get those cells off except by chemical means.

Gurin May I introduce the disturbing thought that in abnormal livers the sampling is too small, and the variation too large, to justify attempts to do quantitative studies on such samples I realize that many experimenters are attempting to do this, and that it is important On a quantitative basis, however, it is an extremely small sample, and a very small part of an organ that may show great changes. What do quantitative results mean, at best, with such samples? Qualitative changes are fine I am just asking for information, I am not an expert in this field.

Shorr: If one measures four or five things quantitatively, their relationship to one another may constitute a frame of reference. In other words, one should not be too discouraged

Best: Dr Kosterlitz, would you continue, please.

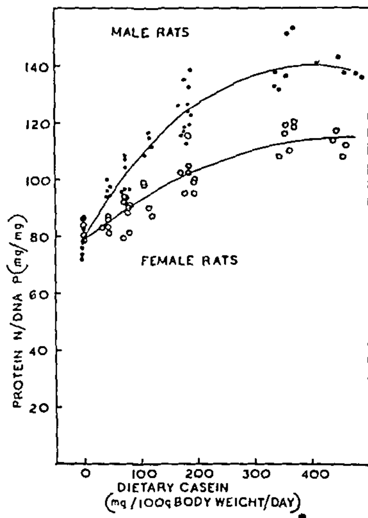


FIGURE 20 The protein contents of liver cells of male and female rats fed on diets containing varying amounts of casein The lines were drawn from regression equations

Kosterlitz. The next section of this talk deals with dietary effects on the composition of the liver cell. If we analyze the protein content, expressing it as shown in Figure 20, in mg protein N per mg DNA P, we find that with increasing protein intake, in this case casein, the protein content of the individual liver cell goes up,

Lundsgaard: How long are the experimental periods in this experiment?

Kosterlitz: A week, in each case.

Shorr: Does it make any difference whether the caloric differences are with respect to fat or carbohydrate?

Kosterlitz: We did not go into that very carefully. We did a few experiments and it did not seem to make very much difference, but I would not like to commit myself on this point.

Vars: Calories, whether they came from fat or carbohydrate, or by feeding isocaloric and isonitrogenous diets, did not make a great difference in liver protein regeneration after partial hepatectomy (32). These studies were done with 3, 10 and 30 per cent fat diets

Kosterlitz: In order to maintain the same amount of liver protein, as the energy intake is reduced, we have to increase the protein intake very considerably, relatively much more than we should expect from the reduction in energy intake (Figure 21). For instance, if we reduce the energy intake by 50 per cent, the dietary casein required to produce equal amounts of liver protein has to be about fourfold. That would mean that when we are confronted with the problem of what we shall give, either protein or calories, calories should have preference, because it is much more expensive to produce the same amount of liver protein by increasing the protein concentration

Shorr. Are we not getting into specific dynamic action of proteins?

Kosterlitz· I should not like to commit myself on that point *However, I think our findings agree with what I know* of results obtained by balance experiments Elman and his collaborators (33), in experiments on dogs, found exactly the same thing in *balance experiments.*

Gyorgy Do these results apply to all kinds of protein, or did you work only with casein?

Kosterlitz: That was done only with casein

Gyorgy· The problem you raised is a very interesting one Last year at the FAO/WHO meeting of nutritional experts in Gambia (34), the Uganda group (*J N. P. Davies, H. C. Trowell*) claimed that in kwashiorkor, a disease of protein malnutrition, the major etiological factor is not a low protein intake *per se*, but a low protein ratio to total caloric intake. In other words, *caloric* undernutrition should not be as harmful as relative protein undernutrition, at least with regard to the development of kwashiorkor Trowell even devised a tentative formula: *With from 15 to* 20 per cent of total caloric intake supplied by protein, there would be no

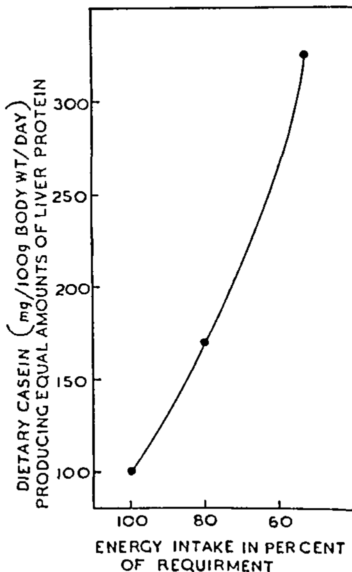


FIGURE 21 The amounts of dietary casein required to give equal amounts of liver protein when the caloric intake was reduced below requirement The line was drawn by eye

kwashiorkor. If there were from 10 to 15 per cent, a danger zone would be created, and with only 10 per cent, or under, the result would be kwashiorkor.

However, this formula is not in accord with what we know of protein metabolism; I refer you to the work of Munro (35). All experimental and clinical observations support the thesis that with higher caloric intake, protein utilization, even with subnormal protein supply, is better than with caloric deficit.

Goldblatt: Would that depend on the kind of protein?

György: That is the reason I raised the question; the first difference may be between animal and vegetable protein

Watson: Did you not suggest that yourself with relation to milk?

Gyorgy: Yes The "Uganda formula" is at variance with the *analysis of human milk in which the ratio of protein calories* to total caloric content is from 6 to 8 per cent. Thus, human milk should be one of the best suited foods to *produce* kwashiorkor However, on the contrary, everybody agrees that human milk is the best food to *prevent* kwashiorkor. Human milk may be an exception, for reasons not yet known, or perhaps the Uganda formula is not quite correct. In addition, the type of protein, and even that of carbo*hydrate*, may be important. *In Africa manioc, sorghum, millet*, and corn are the source of carbohydrate. There is much less kwashiorkor in rice-eating countries.

Shorr: Are there any changes in the over-all metabolic rates of the patients under these different diets? In other words, do we force a higher metabolic activity by higher calories, and hence accentuate a deficiency?

Gyorgy: That would be the simplest explanation for all vitamin deficiencies We know that vitamin deficiencies do not occur in starvation Rickets will not develop in starvation, and this applies *to almost all types of deficiency* conditions. However, in kwashiorkor we are dealing supposedly only with protein nutrition, which should improve with higher caloric intake.

Kosterlitz However, is there not a difference between this type of experiment, where there is a change in protein only, and in field work, where there is a change in so many other factors, such as vitamins, and so on?

Gyorgy: *That was the reason I asked* you whether you used only casein, and what types of carbohydrates were present in your rations?

Kosterlitz The carbohydrate used in the experiments was either sugar or corn starch. The supply of vitamins was *kept constant*;

that is an important point. They were given separately in small dishes before the rats had their food. The ones with a reduced caloric intake had as much of the vitamin B complex as those with a high intake. That would not be the case in field experiments.

Best: Dr. Gyorgy, if the carbohydrate were cut down, leaving the protein the same, then kwashiorkor would not develop?

Gyorgy: This is correct.

Shorr: Doesn't that raise the possibility that lower calories may reduce the over-all metabolism, which is the reverse of which you said?

Gyorgy. With regard to protein retention, it is generally claimed that with higher caloric intake, protein retention will improve This should reduce the danger of protein deficiency

Hoet. I did hear a little about this from Dr. E J. Bigwood,* who was at the meetings in Gambia. But I do not think they all agreed as to the causes of kwashiorkor. However, it is a fact that when children of one to six years take nothing but manioc, which contains zein, with milk or milk powder, it prevents the appearance of some of the most important kwashiorkor symptoms Those children can eat as much as they like, it does not matter If they come to the hospital and get milk powder, it can prevent the disease Thus, I believe that a reduction in the large amount of calories taken under the form of manioc is of no use It is protein that must be supplied It must be given even if the natives are going to eat a good dinner of manioc, which they like

Gyorgy Manioc has only about two per cent protein, and is practically free from sulfur-containing amino acids Such a milk campaign is being carried out very successfully in the Belgian Congo, under Government supervision

Hartroft I wonder whether age is not an important factor in these considerations In the disease, kwashiorkor, children and infants are chiefly affected In the experiments just described, did Dr Kosterlitz use adult rats?

Kosterlitz Yes

Hartroft Have you compared such curves for weanling rats?

Kosterlitz No

Hartroft I wonder if quite different results might be obtained by using young animals in active stages of growth

Goldblatt Are you not comparing experiments of rats with a complete dietary deficiency, with the chance diet of natives about

* Personal communication

which you know nothing? It seems to me that is the crux of the situation.

Kosterlitz: When people take too many calories of a low-protein diet, their appetite will be satisfied even though they have not taken in sufficient protein. However, in our experiments on the rats the point at issue was: what is the effect of reducing the caloric intake *below requirements? The natives took calories up to requirements*, which was an entirely different situation.

Gyorgy: No; the calories were often below requirements.

Shorr. Do we not have to determine protein sparing ability in relation to disease? For instance, if the minimum wear-and-tear protein is reduced to low levels, as occurs in Graves' disease, we have to go way up in the nonprotein calories. I think we have a *situation there in which we are trying to relate normal standards* as to protein sparing to a disease process. I believe we shall only obtain valid data if we actually do quantitative metabolic experiments There must be a minimum protein requirement in any specific experimental situation below which, regardless of what we do, we are not able to keep an animal in positive balance.

Kosterlitz: I think Dr Hartroft has probably put his finger on the heart of the problem; namely, that these are *adult rats*, and you are dealing with growing children I should like to quote again the experiments of Ely and Ross (7), who fed young rats on protein-free diets They found an increase in the DNA content of the liver nuclei That will not happen in adult rats. Thus, a similar deficiency may have quite a different effect in young and in adult animals. I think you are quite right: in order to find out more about *this, one would have to put weanling rats on this type* of diet for sufficiently long periods so that there would be interference with normal growth.

Best: All the experimental work on fatty livers with respect to the relation of caloric intake to protein deficiency supports conclusions of the Uganda group If I may just mention very briefly our work on feeding alcohol, our paper (36) was challenged by Dr *Klatskin (37), who thought we had not established* the fact that the increased calories provided by alcohol created an additional demand for choline We have now done all the extra experiments he suggested, and the results are just the same. The increased caloric intake, due to the alcohol, precipitates a condition that is completely controlled by giving choline.

Bollman: May I return to the question of standards? It seems *to me that again we would have to have a basis of reference*, and

I was wondering about the energy intake in relation to the energy requirement, as indicated in Figure 24. How did you calculate that energy requirement; that is, on the 60 per cent intake? Is that on the same basis as the 100 per cent intake? In other words, you have a varying standard there, I suspect.

Kosterlitz. In Figure 23, we have three curves. the upper curve represents the amount of liver protein found when the daily food intake was 7 gm per 100 gm of body weight That is the intake the rat will choose on its own accord The second curve is obtained from rats receiving about 80 per cent of the energy intake of the first group The last curve shows the liver protein of rats whose energy intake was restricted by 50 per cent. The curve in Figure 24 was directly derived from these results.

Bollman The point I am making is that the metabolic rate is down You have not allowed for it in Figure 24

Kosterlitz. I think one could always say that the metabolic rate was going down for the first three or four days The value is the mean value of the whole curve Of course, the situation becomes worse then, does it not?

Bollman. Yes

Kosterlitz The energy requirements are lower. The curve would be even steeper than in Figure 24

Bollman It strikes me that it would be the other way around

Kosterlitz No You see, the energy requirements are lower Therefore, the amount of energy they obtain in percentage of their requirements is higher At least, I think so. Thus, the curve is even steeper

Bollman Actually, in the energy requirements of the rat, for instance, where you have 60, it really ought to be over about where the 80 is, but at a different level In other words, when the food intake is cut down to 60 per cent, with a 100 per cent energy requirement, the animal compensates for that and cuts down his energy requirement, so that at the level of 60, you are still feeding him about 80 per cent of the calories he is using at that time

Kosterlitz That is right Thus, the curve would be actually steeper

Bollman I shall have to figure that one out

Shorr Are there no nitrogen balances?

Kosterlitz No nitrogen balance experiments To date, we have found that the composition of the liver cell so far as its protein content is concerned, is a direct function of the dietary protein intake In Figure 25 we have, on the ordinate, lipid P per mg

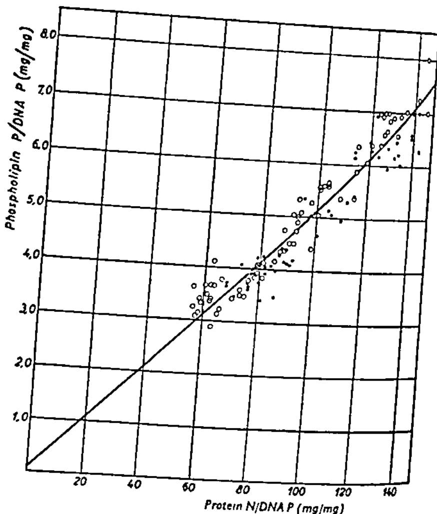


FIGURE 25 The relationship between the phospholipid P, and protein N, contents of the liver cells of rats, fed on equicaloric diets containing varying amounts of casein and fat The open circles represent rats on a 40 per cent fat diet, and the dots rats on a 10 per cent fat diet The line was drawn from a regression equation. Reprinted, by permission, from Campbell, R M, and Kosterlitz, H W The effects of dietary protein, fat and choline on the composition of the liver cell and the turnover of phospholipid and protein bound phosphorus *Biochim et biophys acta* 8, 661 (1952)

DNA P, and on the abscissa, protein N per mg DNA P. The phospholipid content of the liver cell goes up with increased protein content of the liver cell, and also with increased dietary protein intake (4,6). The open circles in the figure represent animals which were fed on a 40 per cent fat diet, and the dots, animals fed on a

10 per cent fat diet. The effect of the fat content of the diet is very small. It is, as a matter of fact, statistically significant. However, the phospholipid content of the liver cell is, in the first instance, determined by the dietary protein intake and not by the fat intake of the diet Further, the phospholipid content of the liver cell is quite independent of whether choline is present in the diet or not (6).

Best: You have methionine in the protein.

Kosterlitz: Yes

Best: Therefore, you had available choline

Kosterlitz: Yes, but some of these animals were fed on an 8 per cent casein high-fat diet without choline, and had very fatty livers; supplementation with choline, without any other change in the diet, reduced the triglyceride content, but there was no alteration in the phospholipid content It was the choline in the diet, even allowing for the methionine, which was definitely below the requirement

Best However, you changed the available choline when you changed the protein

Kosterlitz That is correct.

Best Thus, the statement that choline had nothing to do with it might be modified?

Kosterlitz Yes, but on the other hand, I have animals at the lower end of this curve with a very low protein and high choline intake, and animals at the upper end of the curve with no choline but a high protein intake, so that probably the two amounts of choline which are available here and there, may be of a similar order

Best There are a lot of variables there The caloric intake comes in too, does it not?

Kosterlitz Yes, and in such a way that the protein content of the liver cell decreases with reduced caloric intake, and the phospholipid content goes down with it

Best These were adult rats?

Kosterlitz Yes

Gyorgy What was the duration of the experiment?

Kosterlitz From two days to four weeks

Gyorgy There was no difference between two days and four weeks in the plotting?

Kosterlitz No, there was no difference The phospholipid content and the protein content of the liver cell, were always very closely correlated

Best: That is a very important and startling statement.

Kosterlitz: I think if we analyse all the data which are available in the literature, we shall always find that the determining factor for the phospholipid content of the liver cell, that is phospholipid P related to DNA P, not its concentration, varies with the protein intake. I have been looking through almost all the available papers, and there is no contradiction to that I do not know how many rats are represented in Figure 25, but we have done it with hundreds of animals. This close correlation between phospholipid and protein is always found * This was confirmed for the rat liver by Davidson (3) and found also for cultures of explants of chick heart embryo by Davidson and Leslie (39).

Best: We have debated this many times over the years in these meetings. The problem that worries all of us is that we should like to have just enough methionine available for other than lipotropic activity, keep it at that level where it satisfies all other requirements except that of making choline. However, we are not sure that this is possible. You said the choline content of the diet, but if you mean the availability of choline in the body, it is something different. However, that is not a big point in your argument at the moment.

Kosterlitz: No, it really is not. Of course, that would lead us almost into the whole problem of phospholipids in relation to lipotropic factors

When I first found this relationship, I was rather worried about it, as a matter of fact, I delayed publication of it However, my personal interpretation of it, as you will see later, is that dietary protein not only changes the protein content, but the whole cytoplasm of the liver cell. It is not a protein storage, but the cell becomes either larger or smaller as the dietary protein goes up or down.

Figure 26 gives us further evidence. If it were only phospholipids one might perhaps be worried about the situation, but we found the same relationship between ribonucleic acid (RNA) and liver protein (4) As the protein content of the liver cell goes up, so does the RNA content Thus, we really have three important constituents of the liver cell; namely, protein, phospholipid, and RNA

* It should be noted that, while protein N and phospholipid P are closely correlated, choline may cause changes in the nitrogenous base of the phospholipid molecule For instance, Fishman and Artom (38) found, after choline administration an increase in the choline-containing fraction at the expense of the other fractions

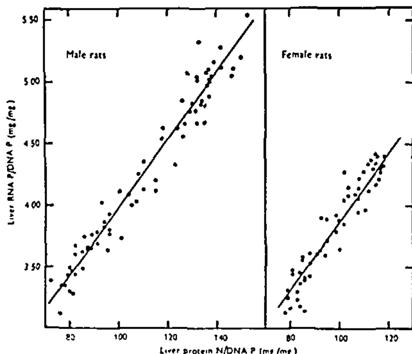


FIGURE 26 The relationship between the RNA P, and the protein N contents of the liver cells of rats, fed on equicaloric diets containing varying amounts of casein The lines were drawn from regression equations Reprinted, by permission, from Campbell, R M and Kosterlitz, H W The effects of growth and sex on the composition of the liver cells of the rat *J Endocrinol* 6, *308 (1950)*

which with increasing dietary protein go up, and with decreasing dietary protein go down

As you see, the relationship can be expressed by a linear regression equation, but the line does not go through zero The scatter around the line is so small that we can use this regression equation to determine whether there is any RNA in the liver cell, which is not correlated with its protein content

Goldblatt When you use the word "protein," should you not, in your statement, include the word "complete"? I ask that because you obviously do not really mean just protein If it were incomplete protein you would not obtain these results, is that correct?

Kosterlitz Oh, yes, you would still get the results. You see, the protein which is plotted on the abscissa is not dietary protein What

we are correlating here is the protein content of the liver cell, with its RNA content. I have previously shown that the protein content of the liver cell is a function: (*a*) of the quantity of protein in the diet, and (*b*) of the quality of protein in the diet. Now we are investigating what happens within the liver under these different conditions, and how we can correlate the various constituents of the liver cells with each other.

Goldblatt: I was thinking about the question of choline content Suppose you had used peanut meal, as opposed to casein, do you think your results would have been the same?

Kosterlitz. I think so.

Best: In other words, a methionine-free protein would give you the same result?

Goldblatt: Yes.

Kosterlitz: Referring to Figure 26 again, I do not think it has anything to do with this particular point at issue, because what we have on the abscissa is not the dietary protein but the liver protein correlated with phospholipid and we have no evidence for the assumption that the composition of liver protein will vary with the composition of the dietary protein.

Shorr· There is, in other words, a standard "liver stuff"?

Kosterlitz: Yes; the point I should like to make is that there is a mathematical relationship between these liver cell constituents The "liver stuff" as you call it, or the liver cytoplasm, is, in the first instance, determined by the quantity and quality of the dietary protein, and by the amount of dietary fat However, the major determining factor is the quantity and quality of the protein the diet contains.

Best When you say "quality," what do you mean?

Kosterlitz: I mean the amino acid composition

Gyorgy: Was the correlation between the protein content of the cell, ribonucleic acid and phospholipids, determined only with one form of diet?

Kosterlitz. No, that is not quite so In my early work, published in 1947 (40), I did not differentiate between DNA and RNA, but allowing for the constancy of DNA the same relationships were obtained. I published such experiments with rats fed on casein, gelatin and yeast, and have also unpublished data for zein and zein supplemented with tryptophane and with lysine.

In Figure 26, where I show the relationship between RNA and protein in the liver cell, there is a slight difference between the male and female rats The lines are parallel, but in the male rat,

for an identical protein content of the liver cell, there is more RNA (4). So far, we have found that the composition of the liver cell, as I said before, appears to be mainly determined by the quantity and quality of dietary protein The next point I should like to discuss is the rate of synthesis of some of these constituents When one puts an animal on a protein-free diet, the RNA content falls rapidly at first and more slowly later (Figure 27). I shall discuss the kinetics of this later. I should say here that the values of relative specific activity of RNA are not correct as absolute values,

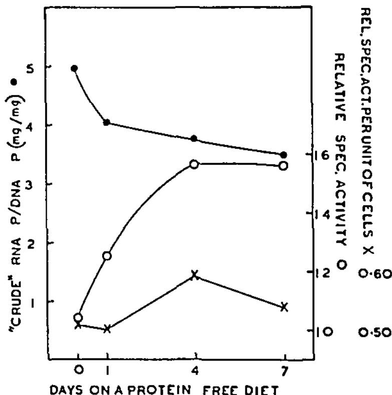


FIGURE 27 The effect of feeding rats on a protein-free diet for varying periods on the "crude" RNA P content of liver cells (• •) the relative specific activity of crude RNA (o o), and the relative specific activity present in a given number of cells i.e. relative specific activity x RNA P per DNA P x 0·01 (x x) "Crude" RNA P is the P present in the RNA fraction prepared by the Schmidt Thannhauser method The specific activities were determined 6 hours after the injection of P^{32}

because they are determined on the crude solvent-extracted protein of the Schmidt-Thannhauser procedure, but this does not affect the general trend of the results very much. The relative specific activity, and therefore the turnover of the RNA, goes up as the RNA content falls, so that with the diminution of RNA molecules in the liver cells, each of the remaining molecules turns over more rapidly. The lowest curve shows the net result; namely, that the total number of molecules formed remains more or less constant. This means that while the amount of RNA in the liver cell is determined by dietary protein, the total amount of RNA formed per unit of time is not (41).

Munro and his collaborators (42) have shown that the rate at which molecules are synthesized is a function of the energy intake of the rat. Figure 28 is a slight modification of the presentation of the results by these authors. In the first group of columns, we have

FIGURE 28 The effects of changing the energy and protein contents of the diet on (first group of columns) the RNA P content of rat liver, (second group of columns) the relative specific activity of RNA P, (third group of columns) the total activity of RNA P (relative specific activity of RNA P × amount of RNA P in the liver) C [illegible] O = protein-free diet High energy intake approx 1[illegible] energy intake approx [illegible] 815 kg cal/sq m [illegible] [illegible]4 hours after the injection of P[illegible] Data [illegible] kramanayake (42)

the amount of RNA in the liver of rats: firstly, on a casein diet, and secondly, on a protein-free diet. The reduction in RNA on the protein-free diet is the kind of effect which I have already shown, it is only found when both diets provide adequate energy intakes. The open blocks, columns 3 and 4, were obtained from rats with reduced caloric intake. You see that the RNA content in both columns is about the same. The results indicate that when the diet contains protein, a reduction in caloric intake has a much greater effect than when the diet is protein-free. I would draw your attention to the fact that in this figure the zero point is well below the basic level The turnover, which is shown in the second group of columns, is very much higher on a protein-free diet than on a casein diet when the energy intake is adequate. That corresponds to what was shown in Figure 27 However, if the caloric intake is low, it does not make any difference whether there is casein present in the diet or not

If we now estimate, not the rate at which each molecule turns over, but the number of molecules that are being turned over per unit of time (third group of columns), we find that what really determines the production of RNA molecules is not so much the casein intake, but the amount of energy supplied by the diet. These results were obtained when the rats were killed 24 hours after the administration of P^{32} In rats killed 4 and 8 hours after P^{32}, the results obtained by Munro, *et al* (42) are not so clearcut.

This agrees very well with the findings of Dr. Bollman and his collaborators (43) obtained some years ago, namely, that the turnover of phospholipid P is higher in animals which have been given thyroxin than it is in normal animals This means that an increase in energy requirements raises the turnover of phospholipid P in the liver

Figure 29 shows something similar In pregnant rats, the phospholipid turnover in the liver increases progressively as the pregnancy goes on If we terminate the pregnancy, or take out the fetuses, which determine the energy requirements, the turnover decreases (44) Thus, if we may summarize the situation as it now appears to us, the protein content, the phospholipid content, and the RNA content of the liver cell, seem to be determined by the dietary protein The rate at which these constituents are synthesized depends on extrahepatic factors, namely, the energy requirements of the body as a whole

Shorr A little while ago you said caloric intake, rather than energy requirements

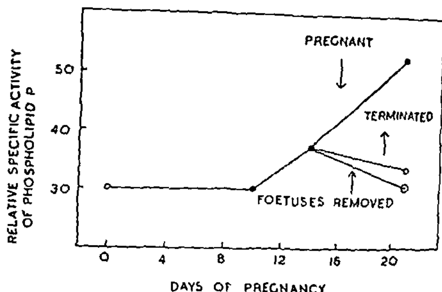


FIGURE 29 The effects of normal pregnancy, or termination or removal of the fetuses on the 14th day of gestation, on the relative specific activity of phospholipid P The specific activities were determined 6 hours after the injection of P^{32}

Kosterlitz. I think that comes more or less to the same thing If we keep a rat on energy intake, which is reduced by 50 per cent for a period of a week or longer, the energy requirements also will be low

Shorr. If you go long enough, do you ever get a point at which the increase in turnover does not quite compensate, and is that a critical breaking point for the liver? I am speaking of the relationship between the increased turnover, the decreased total amount fed, and effective function of the liver cell.

Kosterlitz I do not think we have gone as far as that.

Gurin. Do you imply that an increased turnover in these instances represents the summation of continuous steady synthesis of something like RNA, but a reduced total amount present in the cell, which, of course, would be responsible for this increased turnover, as you call it? On a reduced intake of food, would you say there is a decreased amount of RNA in the cytoplasm?

Kosterlitz In an animal fed on a diet containing protein, when the food intake is reduced the am[illegible] of RNA in the cytoplasm goes down Because the RNA is [illegible] n this way, the turnover rate does not go [illegible] the othe[illegible] ie RNA in the liver cell is reduced b[illegible] it on a [illegible] diet with adequate

caloric intake, the amount of RNA goes down, but the turnover rate goes up. Thus, the total amount of RNA produced is almost as high as before.

Shorr. Do you mean this is actually new synthesis or turnover? I mean, is it the same number of molecules turning over faster, or are you creating more molecules?

Kosterlitz. It is an incorporation of P^{32}.

Shorr. That would not differentiate it.

Kosterlitz: I am not quite sure what your point is.

Shorr: Unless you know the nature of the entire cycle, or its utilization, you cannot tell whether it is new formation of a total molecule, or whether – inasmuch as you are measuring phosphate – it is a turnover cycle of the nucleic acid.

Kosterlitz. It is only the phosphorus. Now I come to the next point, on which I should like to have the opinion of this group. That is, the significance of these changes in liver protein.

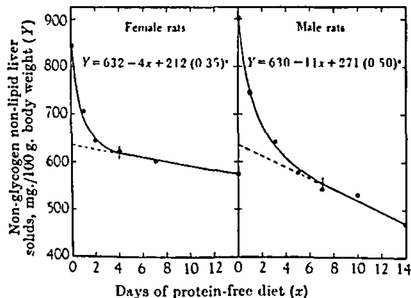


FIGURE 30. Rapid and slow loss of liver cytoplasm in rats fed on a protein-free diet. Influence of sex on the rate of disappearance of labile liver cytoplasm. The lines were drawn from regression equations. Reprinted by permission from Campbell, R. M., and Kosterlitz, H. W.: The influence of sex on liver cytoplasm. *J. Physiol.* 105, 33P (1946) (Cambridge Univ. Press).

When an animal, fed on a normal diet, is transferred to a protein-free diet, liver protein is lost very rapidly, as has been shown by Addis and his collaborators (45), and the rate at which it is lost is faster in female than in male animals (Figure 30). From a statistical analysis, it would appear that we have two significantly different rates of loss of liver protein: first, a fast one, which is the exponential component of the regression equation (46), and then a slow linear one. At the same time, with the loss of protein, there is also a loss of phospholipid and RNA. This difference in rates of loss induced me to call that part of the liver cytoplasm which is lost rapidly, "labile liver cytoplasm." I do not wish to imply by that term anything more than a description of the kinetics (40), nor do I wish to say whether this part of the cytoplasm is labile metabolically or structurally. I do not know whether its composition is different from the cytoplasm which is lost at a later stage of protein deprivation

As you all know, after an animal has been on an adequate diet, if you place it on a protein-free diet, during the first two or three days it will excrete nitrogen in the urine which is in excess of the basic amount excreted during the later stages of the protein-free regimen Figure 31 shows the excretion of extra nitrogen which

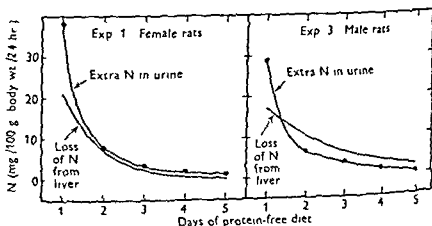


FIGURE 31 Excretion of urinary N in excess of basal N, and loss of N from the liver during the first few days of a protein-free regimen The curves for "extra N" were fitted by eye, and those [illegible] regression equations obtained from other [illegible] and Kosterlitz, H W The [illegible] and urinary nitrogen excretic [illegible] Press)

is compared with the nitrogen loss from the liver. The similarity is rather striking. The amount of nitrogen lost from the liver accounts for about 60 per cent of the extra nitrogen appearing in the urine (47)

Shorr. Did you, by any chance do sulfur analyses?

Kosterlitz: No we did not Thus, the nature of the labile liver cytoplasm accounts for a great percentage of the urinary nitrogen. The important point, as Dr. Vars probably will tell us later, is that enzymes are also lost

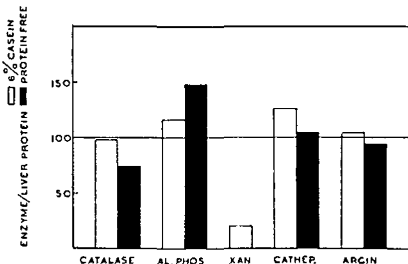


FIGURE 32 Changes in the enzyme contents of livers of rats fed on a 6 per cent casein diet for 3 weeks (xanthine oxidase for 6 days) or a protein free diet for 2 weeks Al phos alkaline phosphatase xan xanthine-oxidase, cathep cathepsin, argin arginase The ordinate gives the ratio of enzyme to liver protein (100 = values found in rats fed on a 25 per cent casein diet) The loss of liver protein was approx 23 per cent on the 6 per cent casein diet and approx 40 per cent on the protein free diet Compiled from data by Miller (48)

Figure 32 was prepared from values reported in the paper by L. L. Miller (48), in which he showed the loss of catalase, alkaline phosphatase, xanthine-oxidase, and arginase They are lost at almost the same rate as the proteins, with the exception of xanthine-oxidase The latter enzyme is of particular interest, because the time relations of the losses of xanthine-oxidase, and the labile liver cytoplasm, seem to be very similar

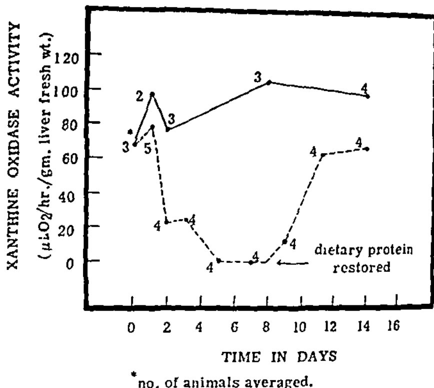


FIGURE 33 Variations in activity of liver xanthine-oxidase of rats fed on a normal ration or a nonprotein ration with time after placing the animals on the respective rations Solid line rats fed on 18 per cent casein + 0 25 per cent DL-methionine ration Broken line rats fed on a nonprotein ration Reprinted, by permission, from Litwack, G, Williams, J. N, Jr, Feigelson, P., and Elvehjem, C A Xanthine oxidase and liver nitrogen variation with dietary protein *J Biol Chem* 187, 605 *(1950)*

Figure 33 has been taken from a paper of Litwack, Williams, Feigelson, and Elvehjem (49). You see that xanthine-oxidase, the lower dotted curve, is completely lost within four to five days on the protein-free diet, and is almost fully re-formed after the dietary protein has been restored for two days.

In Figure 34 we shall see what happens to labile liver cytoplasm when rats are fed on a protein-free diet. Here we have the starting point, in four days, all the labile cytoplasm is gone Refeeding with a protein diet restores it in two days (31). Thus, it would seem that there might be a difference between the labile part of the cytoplasm, and the remaining part. However, the question still is· is it labile metabolically or structurally? This is a rather important point,

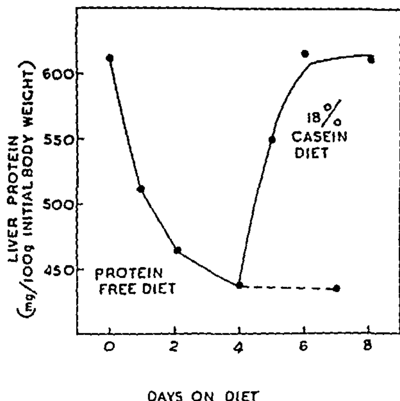


FIGURE 34 Rate of breakdown and formation of labile liver cytoplasm The lines were fitted by eye

because if the first alternative were correct the protein might be an emergency reserve, and also the phospholipid and the RNA However, we have not found any evidence of metabolic lability

Figure 35 shows experiments in which the protein and RNA contents of liver cells were estimated after administration of carbon tetrachloride We thought that the damage caused thereby should lead to a breakdown of liver cytoplasm if it were metabolically labile (50,51) On the left half of the figure we have the protein contents of the liver cells of rats fed on a 70 per cent casein diet After carbon tetrachloride, there was no significant change As a matter of fact, the new cells formed after carbon tetrachloride poisoning have a protein content which depends on the protein content of the diet in the same way as it does for normal cells Furthermore,

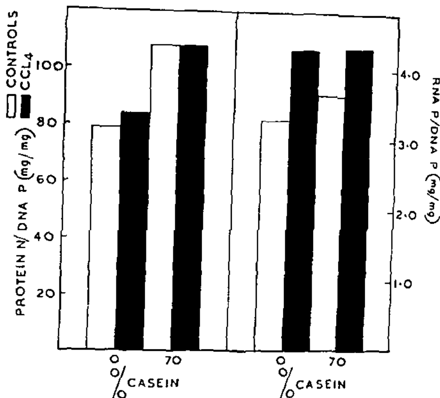


FIGURE 35 The protein and RNA contents of liver cells of rats 24 hours after a single subcutaneous injection of carbon tetrachloride (0 2 ml per 100 gm of body weight) The diets contained either 0 or 70 per cent casein, the fat content was 10 per cent

if the labile liver cytoplasm were metabolically active, the turnover of liver phospholipids, or RNA, should be greater when labile liver cytoplasm is present than when it is absent.

I have shown that this does not hold for RNA (Figure 27). Figure 36 shows the same facts for phospholipids When animals on diet adequate in protein and calories are transferred to protein-free diets, the phospholipid content comes down and its turnover goes up (41) In other words, the turnover of the phospholipid P content of the remaining cytoplasm, which has lost the labile component, is more rapid than that of livers with labile cytoplasm Therefore, the turnover of the labile portion cannot be higher than that of the remaining portion

Evidence was presented by Solomon and Tarver (52), which they thought would show metabolic lability of the protein moiety

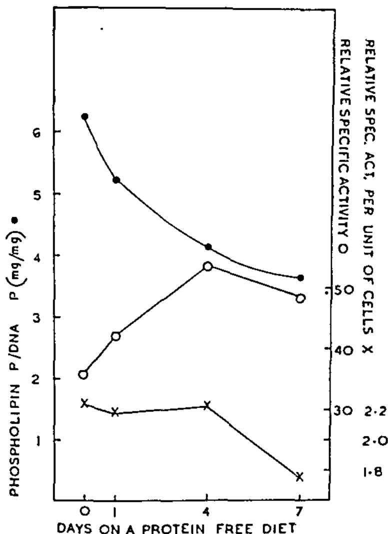


FIGURE 36. The effects of feeding rats on a protein free diet for varying periods on the phospholipid P content of liver cells (• ——— •) the relative specific activity of phospholipid P (o ——— o) and the relative specific activity present in a given number of cells, i.e. × phospholipid P per DNA P × 0·01 (x ——— x). The specific activities were determined 6 hours after the injection of P

TABLE X

Change in Standard Specific Activity of Methionine Sulfur in Rat Liver Protein

Casein	3 hours	4 days	Per cent loss
0	4.77	3.59	25
25%	1.93	1.14	41
50%	1.27	0.58	54

Reprinted, by permission, from Solomon, G., and Tarver, H.: The effect of diet on the rate of loss of labeled amino acid from tissue proteins. *J. Biol. Chem.* 195, 447 (1952).

of labile liver cytoplasm (Table X). They fed animals on diets with different concentrations of casein, and then injected methionine labelled with radioactive sulfur. They found that three days after injection, there was more radioactive sulfur in the liver proteins of rats fed on a protein-free diet than in rats fed on a 50 per cent casein diet. That, of course, they concluded was simply due to the greater amount of liver protein in the rats on the high-protein diet. The point they really made was that after four days more radioactive sulfur was lost from the animals which had labile cytoplasm, than from animals which had none, and this induced them to assume that the labile part of the cytoplasm turned over more rapidly. There is, however, one weakness in this argument, which is that in animals which are fed on a high protein diet, there is a very definite diurnal cycle of liver proteins. After meals the amount of protein in the liver cells increases, but between meals, and particularly before meals, protein is lost from the liver, and that, of course, will lead to a more rapid loss of methionine sulfur in rats with labile liver cytoplasm, than will occur in those without it. Thus, as far as I can see, there is no evidence of metabolic lability of labile liver cytoplasm. All I can say about its function or significance is that it seems to be an integrating indicator of the state of the protein metabolism of the body. However, I do not know what its function is, and should be most grateful for any suggestions in that respect.

Shorr. This problem of deposit protein has remained unresolved for so many years that it may be worth while reporting even an occasional observation that may indicate a difference in the nature of the protein lost at one stage of a disease and at another. In

observations with Dr. G. Donald Whedon on the excess protein loss during the acute and chronic stages of paralytic poliomyelitis, we noted that the sulfur-nitrogen ratio in the urine was distinctly higher during the acute stage than the 1:14, which is usual and which prevails during the subacute and chronic stage of the disease If you still have the urine specimens, you might see whether any such deviations occurred under your experimental conditions.

Kosterlitz: We actually meant to do that, but there were other problems on hand, and did not do it, I am afraid

Gyorgy. Why do you call it "labile liver cytoplasm"? Would it not be possible that it is just the result of some hormonal influence, say, that of the gluco-corticoids? Protein deficiency causes stress, and then there is a hormonal influence which lasts for two or three days.

Kosterlitz· Little work has been done on the effect of corticosteroids on liver protein, but there have been two papers on rats (53,54), and one on guinea pigs (55) In every instance there was an increase in liver protein even when the animals were fed on a protein-free diet That is the opposite of what we ought to find if stress lowered labile liver cytoplasm

Gyorgy I just mention it as a possible suggestion Perhaps it is not really labile liver cytoplasm but has been reduced by some hormonal influence which you produced by your protein deficiency

Kosterlitz. Even within one day?

Gyorgy. Yes

Shorr: Could this merely be a rate phenomenon in which certain factors have now come in to influence in the direction of slowing the rate of reaction?

Kosterlitz. Yes, I have carefully considered that I have often wondered whether I am justified in distinguishing between the exponential and linear parts of the curve My statistical friends think I am, but I have often been doubtful about it However, the fact that xanthine-oxidase runs so parallel to labile liver cytoplasm seems to indicate that we deal with something more than just a rapid rate at first, and then a slowing down

Shorr. Could this be one of the ways in which hormones act to prevent protein loss? Perhaps you are actually providing the raw data for understanding the mechanisms by which ACTH, or pituitary hormones, inhibit the rate of carbohydrate oxidation

Kosterlitz The real trouble as far as I am concerned, is that hypophysectomized and adrenalectomized rats can have labile liver cytoplasm provided that the hypophysectomized animals have been

TABLE X

Change in Standard Specific Activity of Methionine Sulfur in Rat Liver Protein

Casein	3 hours	4 days	Per cent loss
0	4.77	3 59	25
25%	1 93	1.14	41
50%	1.27	0 58	54

Reprinted, by permission, from Solomon, G, and Tarver, H · The effect of diet on the rate of loss of labeled amino acid from tissue proteins *J Biol Chem* 195, 447 (1952)

of labile liver cytoplasm (Table X). They fed animals on diets with different concentrations of casein, and then injected methionine labelled with radioactive sulfur. They found that three days after injection, there was more radioactive sulfur in the liver proteins of rats fed on a protein-free diet than in rats fed on a 50 per cent casein diet. That, of course, they concluded was simply due to the greater amount of liver protein in the rats on the high-protein diet. The point they really made was that after four days more radioactive sulfur was lost from the animals which had labile cytoplasm, than from animals which had none, and this induced them to assume that the labile part of the cytoplasm turned over more rapidly. There is, however, one weakness in this argument, which is that in animals which are fed on a high protein diet, there is a very definite diurnal cycle of liver proteins. After meals the amount of protein in the liver cells increases, but between meals, and particularly before meals, protein is lost from the liver, and that, of course, will lead to a more rapid loss of methionine sulfur in rats with labile liver cytoplasm, than will occur in those without it. Thus, as far as I can see, there is no evidence of metabolic lability of labile liver cytoplasm All I can say about its function or significance is that it seems to be an integrating indicator of the state of the protein metabolism of the body However, I do not know what its function is, and should be most grateful for any suggestions in that respect.

Shorr This problem of deposit protein has remained unresolved for so many years that it may be worth while reporting even an occasional observation that may indicate a difference in the nature of the protein lost at one stage of a disease and at another. In

observations with Dr. G. Donald Whedon on the excess protein loss during the acute and chronic stages of paralytic poliomyelitis, we noted that the sulfur-nitrogen ratio in the urine was distinctly higher during the acute stage than the 1.14, which is usual and which prevails during the subacute and chronic stage of the disease. If you still have the urine specimens, you might see whether any such deviations occurred under your experimental conditions

Kosterlitz: We actually meant to do that, but there were other problems on hand, and did not do it, I am afraid

Gyorgy Why do you call it "labile liver cytoplasm"? Would it not be possible that it is just the result of some hormonal influence, say, that of the gluco-corticoids? Protein deficiency causes stress, and then there is a hormonal influence which lasts for two or three days

Kosterlitz Little work has been done on the effect of corticosteroids on liver protein, but there have been two papers on rats (53,54), and one on guinea pigs (55) In every instance there was an increase in liver protein even when the animals were fed on a protein-free diet. That is the opposite of what we ought to find if stress lowered labile liver cytoplasm

Gyorgy I just mention it as a possible suggestion Perhaps it is not really labile liver cytoplasm but has been reduced by some hormonal influence which you produced by your protein deficiency

Kosterlitz: Even within one day?

Gyorgy Yes

Shorr Could this merely be a rate phenomenon in which certain factors have now come in to influence in the direction of slowing the rate of reaction?

Kosterlitz Yes, I have carefully considered that I have often wondered whether I am justified in distinguishing between the exponential and linear parts of the curve My statistical friends think I am, but I have often been doubtful about it However, the fact that xanthine-oxidase runs so parallel to labile liver cytoplasm seems to indicate that we deal with something more than just a rapid rate at first, and then a slowing down

Shorr Could this be one of the ways in which hormones act to prevent protein loss? Perhaps you are actually providing the raw data for understanding the mechanisms by which ACTH, or pituitary hormones, inhibit the rate of carbohydrate oxidation

Kosterlitz The real trouble, as far as I am concerned, is that hypophysectomized and adrenalectomized rats can have labile liver cytoplasm provided that the hypophysectomized animals have

force-fed There is no significant difference in the labile liver cytoplasm content between force-fed hypophysectomized and non-hypophysectomized animals, with the exception that hypophysectomized animals have a little less RNA.

Shorr: What about the castrated male?

Kosterlitz: The castrated male becomes very similar to the female.

Shorr: Thus, you have already shown one hormonal influence

Kosterlitz: However, I pointed out at the very beginning that there is a sex difference.

Shorr: Then this would suggest some form of hormonal influence.

Kosterlitz: However, so far as testosterone is concerned, we know it has a protein-sparing effect, increases protein anabolism. What the liver does is only to indicate what has happened to protein metabolism.

Best: Testosterone does nothing in the absence of insulin Did you take out the pancreas?

Kosterlitz: No.

Vars. I should like to make a few comments on the labile liver protein If we go back to some of the early work of Luck (56), using the classical method of attempting to fractionate proteins, we see that the proportion of albumin, globulin, — in the case of the four major fractions — did not change whether the animal was starved of protein, or whether he was well fed This was an indication that the nature of the protein, as they talked about it at that time, did not change in proportion.

In our work with hepatoxic agents (57,58), we made observations similar to those Dr. Kosterlitz mentioned. In situations where we obtained severe degenerative necrosis of varying degrees, we also noted increased amounts of protein in those livers. Taking into consideration the work of Miller and Whipple (59), and others including our own, which indicated that protein did have a protective effect in preventing degenerative necrosis, it seemed anomalous that this liver, which had already been hard hit, should show the same increase in protein after this took place. Here were two situations that were both giving us protein increase, and they led us to doubt whether protein *per se*, the total amount per organ — and [illegible]

metabolic function That is why, in our more recent work, we have been happy to have Dr Otto Rosenthal as an active collaborator, we have turned our attention to trying to define these

changes in protein by measuring total enzymatic activity of certain enzymes in the organ.

Of course, that raises many questions, particularly if we refer to the work of Miller (60), Schultz (61), and Muntwyler (62). With complete inanition, there is a concordance in the drop in alkaline phosphate activity with liver protein loss in short-term starvation experiments If one limits protein only by feeding a protein-free diet, the total nitrogen loss of the liver is the same as in complete starvation, yet the total alkaline phosphatase activity increases from 25 to 50 per cent. Thus, here is one instance in which loss of total protein does not lead to a similar pattern of enzyme protein loss. At least one of the multitudinous enzymatic activities taking place there does not follow. Other instances like that may be cited, particularly the work of Westerfeld (63), at Syracuse, and of Williams (64), at Wisconsin, upon xanthine-oxidase Williams has worked on xanthine-oxidase activity of the liver with relation to amino acid starvation. He has data showing that it is related to specific methionine deficiency, and that by adding methionine to the protein-free diet, there is a return of xanthine-oxidase activity That has recently been complicated still further by the observation (65) that molybdenum also will return xanthine-oxidase activity in the absence of giving additional methionine at that point

Thus, while we like to think we are progressing toward the understanding of metabolic function by the analysis for specific enzymes, that work is still fraught with many unsolved problems in terms of inhibitors, accelerators, cofactors, and things of that nature I think we probably have two strikes and three balls against us and have not really started for first base

Popper. Dr Vars, I know from your previous papers, and you have said it again just now, that the phosphatase activity in tissue or serum does not run parallel to that of other enzymes in liver damage or inanition

Vars I was speaking there only of complete starvation versus protein starvation

Popper In investigations of Drs Huerga and Koch-Weser in our laboratory (66), we were puzzled by the contrast between alkaline phosphatase and esterases, or some dehydrogenases, because in liver damage the phosphatase activity rises in serum and tissue, while that of the other enzymes decreases Could that peculiar behavior be due to the fact that according to histochemical analysis, alkaline phosphatase in most species is not found in the liver cells themselves, but in the wall of the sinusoids, in the

Kupffer cells, and in the cholangioles, while apparently the other enzymes referred to are within the liver cells?

Vars: Alkaline phosphatase is found in both the cytoplasmic and particulate fractions of the liver, when the Schneider procedure is used. Of course, fractionation may lead to artefacts in concentration or adsorption.

In further work of Dr. Rosenthal (67,68), we find evidence that two types of alkaline phosphatase, distinguished by differential sensitivities to cyanide and magnesium ions, exist in liver. They do not change in a parallel fashion under diverse situations of metabolic activity. In complete starvation both activities decrease, while in protein starvation alone the total activity goes up while the magnesium sensitive component decreases in accord with the decrease in liver protein. Such changes as these start complicating the picture

Best: I think we must go on with the third section of Dr Kosterlitz's presentation

Kosterlitz. Dr. Vars, Figure 37 is from your paper, written in collaboration with Drs. Gurd and Ravdin (69), which really belongs to the previous section. This is an experiment with which I was very much impressed, showing that the regeneration of liver cells can proceed in the absence of dietary protein That, again, is a point of importance. Labile liver cytoplasm requires dietary protein, but if there is a need for the formation of new cells, liver protein can be synthesized, as has been beautifully shown by Dr. Vars He first kept his animals on a protein-free diet for a fortnight, and then did a partial hepatectomy. There was regeneration of the liver and deposition of liver protein Of course, when the animals had been fed on a diet containing protein, more protein was deposited in the liver I shall welcome correction by Dr. Vars, but I believe the number of liver cells was identical in the two cases Thus, we have more protein in the cells obtained from livers of rats fed on a diet containing protein That is to say, these cells deposited labile liver cytoplasm, whereas this was absent in the rats fed on a protein-free diet.

Best I do not understand the scale. Does that correspond to the amount of labile, or the total protein?

Kosterlitz: The difference between the protein contents of the two types of cells would be the labile protein fraction. Is that correct Dr Vars?

Vars Yes, the lower line represents the loss of protein, that is, starting at 0 6 after two weeks, I think, of prior protein depletion with adequate calories.

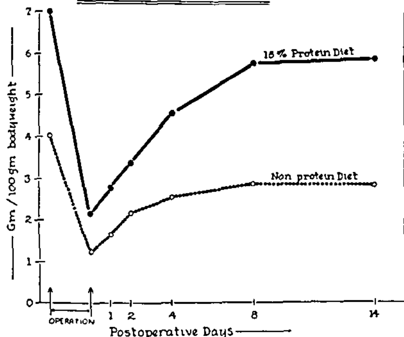


FIGURE 37 Comparison of liver protein values for casein fed, and protein-depleted, rats Values at the beginning and end of operation are shown on left Note that the animals on the nonprotein diet came to operation with the liver protein reduced from 0 70 to 0 40 gm per 100 gm initial body weight as a result of the preoperative period of 14 days on the nonprotein diet Reprinted, by permission, from Gurd, F N, Vars, H M, and Ravdin, I S Composition of the regenerating liver after partial hepatectomy in normal and protein-depleted rats *Am J Physiol* **152, 11** (1948)

Kosterlitz The same thing is often found in carbon tetrachloride poisoning We can remove all labile liver cytoplasm by feeding a rat on a protein-free diet for three or four days If we then inject carbon tetrachloride, new cells are formed with a minimum of protein If we have a similar group of animals on an 18 per cent casein diet, then the liver cells will contain more protein In other words, they contain labile liver cytoplasm

Gyorgy Dr Kosterlitz, you call that which you lose in the first three or four days of starvation "labile liver cytoplasm," but after the first three days, it still goes down

Kosterlitz: It goes down at a very low rate.

Gyorgy: Is the difference here three days plus?

Vars: No, it is two weeks. However, the loss in two weeks is not dissimilar to that which occurs in seven days.

Kosterlitz: The early loss is very considerable. It is 25 per cent of the total protein present, and after that it is very small

The next section deals with a slightly different problem, namely, nondietary effects on the RNA content of the liver. The experiments shown in Figure 38 may perhaps have some connection with

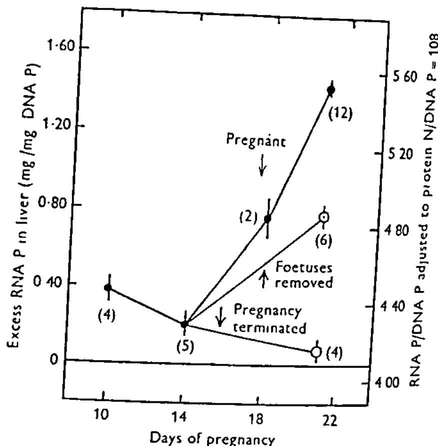


FIGURE 38 Excess of RNA P during pregnancy, after removal of the fetuses, and termination of pregnancy, over values predicted from regression equation for non-pregnant female rats The figures in brackets indicate the number of observations, and the vertical lines twice the standard errors of the mean differences between the observed and predicted values Reprinted, by permission, from Campbell, R M, and Kosterlitz, H W Some effects of pregnancy and lactation on the liver *J Endocrinol* *6, 171 (1949)*

what Professor Hoet discussed earlier, but I do not know. In pregnancy of rodents, i.e., mice, rats, and guinea pigs, the RNA in the liver goes up quite out of proportion to the protein content (44). That is to say, we may or may not have an increase in the protein *content of the liver cell, but the RNA content goes up Histologically,* that can be shown by increased basophilia, particularly in the

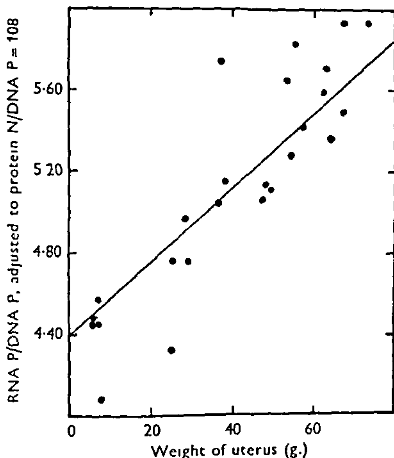


FIGURE 39 The relationship between excess RNA and weight of uteri on the 21st day of gestation The variation of RNA P due to differences in the protein N content of the liver cells has been eliminated by adjusting protein N per DNA P to the mean value of 108 Reprinted by permission, from Campbell, R M, and Kosterlitz, H W Some effects of pregnancy and lactation on the liver *J Endocrinol* 6, *171 (1949)*

periphery of the lobule. It can be abolished by terminating the pregnancy on, say, the 14th day; then the amount of RNA (excess RNA) which cannot be accounted for by the protein content of the liver cell, disappears again. If, on the other hand, the fetuses are removed and the placentae are left in the uterus, excess RNA is still formed. The point at issue here is, what are the factors that cause this increased RNA content of the liver cell, and what is its possible

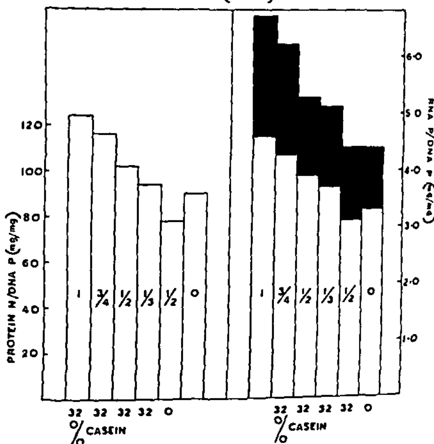


FIGURE 40 The effect of reduction in energy intake during the last 6 days of gestation on excess RNA in the liver of pregnant rats on the 21st day of gestation The columns in the left half of the diagram show the protein content of the liver cells, and those in the right half the RNA content. The clear blocks give the RNA content calculated from the protein content by means of the regression equation, and the shaded blocks represent excess RNA The figures in the middle of the blocks indicate the relative energy intakes of the rats during the last 6 days of gestation 1 being 33 kg cal per 100 gm body weight 0 = 48-hour fast

significance? To cut a long story short, we do not know anything at all about the possible significance, but I think we know something about the factors which are responsible for this increase in RNA

The first factor is the weight of the products of conception, Figure 39 If the excess of RNA is plotted against the weight of the uteri, it is found that these two values are closely correlated (41) We can go further and say that there is a correlation between the weight of the placenta and the excess RNA (70) That is, then, the first factor.

The next factor is the energy intake On the left-hand side of Figure 40, we have the protein content of the liver cells The first column represents animals receiving a sufficient amount of 23 per cent of casein diet to satisfy caloric requirements, which are higher than in nonpregnant rats In the second, third and fourth columns, we find the values obtained from rats receiving three-quarters, one-half and one-third of the requirements As would be expected, we see that the protein content of the liver cell goes down as the food intake is restricted When a protein-free diet is fed which satisfies only half the energy requirements, then liver protein is even lower than on any of the other diets

On the right-hand side of the figure we have the corresponding RNA contents Each column is subdivided into a shaded and clear part, the clear columns being the values calculated from the regression lines for nonpregnant rats which I showed you earlier In other words, these are the amounts one would expect in nonpregnant animals The black columns indicate the amounts of RNA which are in excess of the expected amounts and are due to the presence of the placentae It is clear that with decrease in energy intake, this excess of RNA becomes smaller The protein content of the diet, on the other hand – and I think that is a very interesting phenomenon – has no effect on excess RNA If we compare the results obtained with the two diets supplying half the energy requirements, we find identical values of excess RNA, the only difference between the two diets being that the one contains protein and the other not

We can express that in a slightly different way as in Figure 41, by plotting excess RNA per gram of placenta against food intake. Strictly speaking, that is not quite correct because, while the relationship between weight of placenta and excess RNA is linear, the regression equation contains an absolute term When allowance is made for this, the conclusions which can be drawn from this figure are not materially affected (70) On the abscissa, we have the food

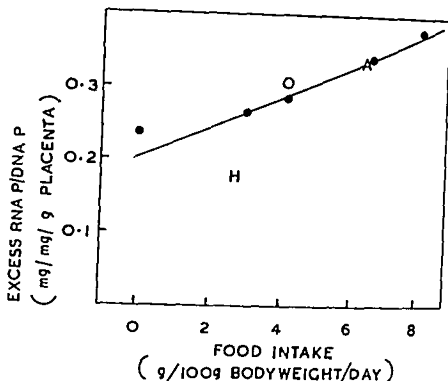


FIGURE 41 The effect of variations in energy intake on the amount of excess RNA P present for each gram of placenta on the 21st day of gestation •——• 23 per cent casein diet 0 protein-free diet for the last 6 days A adrenalectomized. H hypophysectomized on the 15th day of gestation

intake, and we see that the excess RNA is linearly related to the food intake We further find confirmed that the animals on protein-free diet have as much excess RNA as the rats fed on the protein-containing diet Adrenalectomy six or seven days before term has no effect On the other hand, hypophysectomy five days before term does reduce excess RNA, but I do not wish to go into that any further.

A complete statistical analysis of excess RNA shows that the value of RNA P/DNA P in the liver cell of a pregnant rat is conditioned by at least three factors (Table XI). The partial regression coefficient of RNA P per DNA P on protein N per DNA P is not different from that of a nonpregnant animal. Therefore, this part of the RNA behaves in the same way as in a nonpregnant animal There are, however, the two other factors which influence liver RNA in pregnancy, namely, first, the energy intake, and secondly placental weight (70)

TABLE XI

Regression of RNA P per DNA P (21st day) on Protein N per DNA P, Food Intake and Placental Weights. Rats: Normal Pregnancy, or Fetuses Removed on 14th Day, or Hypophysectomized on 15th Day. Diets: 23 Per Cent Casein or Protein-Free

Regression equation

RNA P per DNA P (mg. per 1 mg.) = (0.0330 ± 0.0037) × protein N per DNA P (mg. per 1 mg.) + (0.0615 ± 0.0282) × food intake (gm. per 100 gm. body weight per day) + (0.1300 ± 0.0263) × placental weight (gm.) + absolute term

Absolute terms

Operation	Diet	Absolute term	
None	23 per cent casein	1.16	1.19
None	Protein-free	1.30	
Fetuses removed	23 per cent casein	0.90	
Fetuses removed	Protein-free	1.03	
Hypophysectomized	23 per cent casein	0.77	

Approx. S.E. of differences ± 0.18

Reprinted, by permission, from Campbell, R. M., Innes, I. R., and Kosterlitz, H. W.: The role of hormonal and dietary factors in the formation of excess ribonucleic acid in the livers of pregnant rats. *J. Endocrinol.* 9, 52 (1953).

Such excess RNA is not only found in pregnancy, but also in liver regeneration after hepactectomy, and in liver regeneration due to carbon tetrachloride poisoning. This is seen in Figure 35 which I have already shown in another context. We have there the protein and RNA contents of control livers, and those of animals poisoned with carbon tetrachloride. On the left side are the protein and on the right side the RNA contents. After carbon tetrachloride poisoning in animals fed on a protein-free diet, the protein content does not go up, or only very little, this is the protein content of the liver cell, not the protein content of the whole liver, which of course, goes up. On the other hand, the RNA content per liver cell goes up very significantly. This is an observation which I know has puzzled everybody who has worked on carbon tetrachloride poisoning, namely, that while the histologically visible RNA decreases, the chemically determined RNA increases. However, the important point I wish to make here, is that this excess RNA is produced in

found increases in total nitrogen content of the liver. Unfortunately, wet and dry liver weights, and total nitrogen, were the only factors determined. The presumption might be that if total nitrogen increased, perhaps RNA would have increased also in that situation

Gyorgy: Where was the site of the tumor?

Vars: Subcutaneous.

Hoet: Dr. Wilder Penfield takes out the hypophysis when there are a great many tumors, which considerably slows down the growth of the tumors. Thus, even there it is not quite certain that it is the tissue which is the primary force. It might be the hormonal setting coming from the hypophysis

Vars At present there are a limited number of carbohydrates that we have to consider. glucose, fructose, galactose, and pentoses, be they of animal or of plant origin. In a similar fashion, we have limited the number of fatty acids that are of major importance We have some that are called essential fatty acids, but the range or variety is not great. When we come to abnormal fatty acids, such as Dr. Hartroft mentioned, we begin to get into difficulties when they are not fed in the ratio that we normally employ for our diets In the case of protein, which means "of primary importance" if we go back to the derivation of the word to the Greek word, *proteios*, we have increased our difficulties immensely since we know from Rose's work (72,73) that at least for nitrogen balance in a normal adult, eight essential amino acids are of concern With different species that number increases to ten, and still further Then, to arrive at the permutations and combinations of an excess of one, or a deficiency of another, the problems are multiplied infinitely. One has only to recall the work of Dr. Best, Dr Gyorgy, and others, upon the sulfur amino acids.

That epitomizes, to some extent, the difficulties that Dr. Kosterlitz, and others of us, who have been working with the protein side of the question, have had in ascertaining what is a standard situation, and in many instances we are not very far along the road In fact, it is difficult to establish the norms. We know, at least from Rose's work, that the specific needs for growth and maintenance of these different amino acids vary. We know from enzyme studies that there are profound changes in the relative quantities that one can find in the liver which is ostensibly normal, or protein-depleted, or in the regenerating liver Unfortunately, at the present time, relatively few enzymes are characterized sufficiently in terms of their kinetics, and so on, to make enzyme assays fruitful Thus, the field is wide open for speculation, and probably will be for many years

I think we should welcome some comment from Dr. Madden, because certainly his interest in protein and amino acids has been varied

Madden: I was particularly interested in the comments of Dr Kosterlitz on the labile fraction I feel inclined to believe that in the liver cell cytoplasm it may be a metabolically different fraction, despite the evidence you suggest against this, because it appears to have the differences in enzyme composition already referred to by Drs Kosterlitz and Vars I think this is a point of importance, and one upon which we should place a little more emphasis than upon your suggestion that there are no metabolic differences in the labile and remaining portions of the liver cytoplasm.

As to the suggestion that all the cytoplasm is similar, and that it is a matter of reaction rates as described by the law of mass action, which accounts for a "labile fraction," I would again like to point to the observed enzyme differences shown by Miller, Williams and others, as referred to previously I should like to believe that differences in the activities of the individual proteins in the liver cell indicate that the ones which disappear early under stress may actually be those that are specifically called upon early to meet the stress, and therefore may be qualitatively different There have been other modes of attack on this problem which have suggested the same sort of thing such as the alterations of the urinary nitrogen-to-sulfur ratios during protein depletion

The matter of the relation of the lipid phosphorus content to the protein nitrogen content of the cell I thought was well explained by Dr Vars, in an aside to me, when he said that this was a matter of the relationship of the number of cells, cell surfaces, and particle surfaces to the amount of protein This would obviously be a fairly direct relationship If we increase the number of cells, we also increase the number of phospholipid materials in the surfaces of the cells, and the surfaces of their nuclei and mitochondria By the comment that choline had nothing to do with this you probably meant that the choline content of the diet, within the ranges in which you made the observations did not influence this close correlation between the lipid phosphorus and the protein nitrogen If you had given no choline over a sufficient period of time, and the animals had produced no cells but had just died, you would not have a point on the curve of Figure 25 However, within the range of the normal and abnormal livers which you studied, you do have a rather inescapable correlation between lipid phosphorus and pro-

tein nitrogen. Is that correct, or is this a false interpretation of the curve?

Kosterlitz: I think that probably the most important points on that curve would be the values obtained from animals fed on a protein-free diet, because any choline given to those animals would come from the diet, unless the experimental periods were not long enough, and there were choline still stored in the body.

György: Is methionine in the body?

Kosterlitz: Yes.

György: Methionine forms choline.

Kosterlitz: If we have animals on a protein-free diet – I think three weeks is the longest period in my experiments – with and without choline, the ratio of phospholipid P to protein N is the same in both groups of animals. This may be open to different interpretations, I quite admit that.

Watson. What is the rate of decay of choline? When an animal is placed on a choline-free diet, how long does it take to deplete the choline storage completely? When would you say that you have no choline left?

Best: You never do. It is part of the structure of the body, and it is in every cell. Of course in fasting, as Dr. György said, or when the caloric intake is lowered, the demand is very much less However, we really have to eliminate the methionine before we can say we have eliminated the choline available from the diet

Kosterlitz. However, we cannot do that.

Best: I know, it is a very nice situation

Hartroft Dr. Best, would a fatty liver result from a protein-free, choline-free diet?

Best: I have observed fatty livers on a pure sugar diet.

Kosterlitz Those animals which were on a protein-free and choline-free diet had fatty livers. They had not as much triglyceride in their livers as the rats on the eight per cent casein diet, but they had fatty livers

May I comment on another point which Dr. Madden made? When I said that the liver cytoplasm is structurally and not metabolically labile, I really meant to say that apparently it does not belong to the labile protein pool, which has been described by Dr Whipple and Dr. Madden (74).

Madden And many others before us.

Kosterlitz You are the protagonist I do not know for certain, but I have the feeling that it does not belong to that group. However, it could, qualitatively, I think Although there is a large amount

of protein in the liver, it could account for only a small amount of the labile protein pool of Whipple and Madden

Hanger May I ask Dr. Kosterlitz if he has made any observations on the effects of anoxia? That is a very common factor in liver injury.

Kosterlitz: I am afraid we have not, but it would be very interesting We have been too much taken up with some of these quantitative relationships We wished to obtain as much information on this point as possible before we did any experiment of the type you are suggesting

Gurin· Dr. Kosterlitz, what do you suppose is the source of this extra RNA that you have described here? Have you done any turnover studies? Is this increased synthesis? Is this increased mobilization of materials from other tissues?

Kosterlitz· I am afraid that all my turnover experiments with RNA are open to criticism because I used the RNA fraction of the Schmidt-Thannhauser method, in which there are P-containing compounds other than RNA Nevertheless, there is good reason to believe that this does not affect the general trend of the results

Figure 42 shows the results of the turnover experiments In the upper part the relative specific activity is plotted against duration of pregnancy, and in the lower part we have the relative specific activity multiplied by the amount of RNA P present in a given number of liver cells, whose DNA P content is 1 mg If we look at the upper part first, we find that the relative specific activity is relatively little changed in undisturbed pregnancy, and after termination of the pregnancy on the 14th day, but shows a definite fall when the fetuses alone, and not the placentae, are removed on the 14th day of pregnancy When we now remember that the RNA P content of the liver cell rises during pregnancy, and that this rise is still present after removal of the fetuses but not after termination of the pregnancy (Figure 38) then we find in the lower part of the figure that the amount of RNA P formed in a given number of liver cells in a given time is increased only in undisturbed pregnancy, but not after removal of the fetuses, or of the fetuses and the placentae This means that in pregnancy the turnover per molecule is more or less unchanged throughout pregnancy, at the end of pregnancy there are more RNA molecules in the liver cell which turn over at the same rate as the smaller number of molecules in nonpregnant animals After termination the amount of RNA is back to normal and thus the amount of RNA P formed in a given number of cells per unit time is not higher than in non-

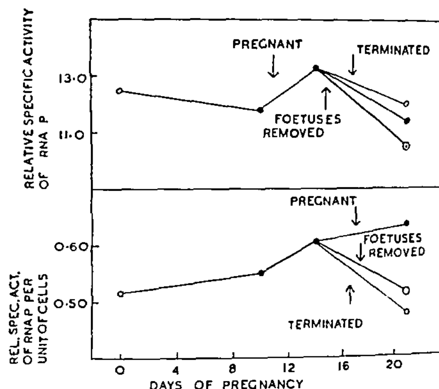


FIGURE 42 The effects of normal pregnancy, termination, or removal of the fetuses on the 14th day of gestation on the relative specific activity of "crude" RNA P, and the relative specific activity of "crude" RNA P present in a given number of cells, i e specific activity × RNA P per DNA P × 0·01 The specific activities were determined 6 hours after the injection of P^{32}.

pregnant rats After removal of the fetuses alone, the amount of RNA remains raised, but because of the decreased turnover rate, the amount formed in a given number of cells per unit of time is about the same as in nonpregnant rats. Thus, the increased amount of RNA P synthesized in pregnancy is due to the increased amount of RNA P in the cell

That is quite different from the case of phospholipid P, as you will remember from Figure 29 There the situation was simpler, because the amount of phospholipid P in the liver cell in pregnancy ... phos-

n

d

the turnover of the available molecules, as indicated by the relative specific activity If we terminate pregnancy or take the fetuses out, the relative specific activity falls to the level of non-

pregnant rats Thus in the case of phospholipids the total amount produced per unit of time is raised because there is an increased rate of turnover, and not because there is an increased amount in the cells In the case of RNA P, on the other hand, the rate of synthesis per molecule remains constant, but there are more molecules.

Shorr. Are you not using synthesis as equivalent to turnover? I think that is one of the things that bothers us

Gurin· You say that the rate of synthesis remains the same, if I understand you correctly. Then you say that the total amount there is greater. Does this not mean that the destruction has therefore been decreased?

Kosterlitz I am sorry, I think we must define the terms The incorporation of P^{32} into the molecule will give us an indication of the rate at which each molecule is renewed Do you agree?

Gurin. Yes. That is a reflection of the rate of synthesis Is this what you mean?

Kosterlitz. Yes, that is one thing

Gurin You say that the rate of incorporation of the phosphorus is a reflection of the number of new molecules that are being produced I think this might be the case, so let us assume that for the time being

Kosterlitz No, I do not think it means that What it really means is the rate at which each molecule present is renewed, or rather, the incidence at which each molecule is renewed

Gurin. I am sorry to quibble about this, but just what do you mean by renewed? You have a molecule of RNA P Is it breaking down and being rebuilt? Is that what you are talking about, or do you mean the synthesis of a new molecule?

Kosterlitz. We have a state of equilibrium, and the more P^{32} is incorporated into that molecule, the more often it has to be renewed during a given time

Gurin I am not sure that you have a state of equilibrium if the total amount is increasing

Kosterlitz This increase is small during the period of six hours elapsing between the injection of P^{32} and the sacrifice of the animal If we have a cell in which there is a large amount of RNA, and another cell in which there is a small amount of RNA, and the relative specific activity of the RNA is identical in the two cells, then the one in which there is more RNA will produce more new molecules of RNA in a given time Do we agree on that point?

Gurin: Yes.

Kosterlitz: All right. That is exactly what happens in pregnancy The incorporation of P^{32} in the molecule is pretty much the same throughout pregnancy, but at the end of pregnancy there is more RNA in the liver cell than in the beginning. Therefore, more RNA is produced at the end of pregnancy.

Best: Thank you, Dr. Kosterlitz: At this point, I think it would be interesting if Dr. Hoffbauer would consent to open a discussion on the extent to which the experimentalists have been able to reproduce or simulate conditions which the clinicians encounter

Hoffbauer: I might begin by citing several areas in which we need considerably more assistance. I regard the number one problem to be the lack of a biological test for the recognition of viral hepatitis. The need for a specific means of detecting this disease is apparent in almost every instance in which the clinician is confronted with a patient who exhibits evidence of acute or chronic liver injury. It seems unlikely that we shall ever determine the role that these viral agents play in the causation of human cirrhosis until such tests have been devised. The inability to transmit the disease to any animal is well known to this audience, it constitutes a tremendous handicap. The experimental studies that have been undertaken in the past were well summarized, in 1949, in the paper by Colbert (75), who has worked with Dr. John Paul at Yale University. To my knowledge there has been no subsequent success in transmitting the agent or agents that cause human viral hepatitis to an experimental animal or to other artificial systems, such as a tissue culture medium This is by no means an index of the lack of endeavor on the part of numerous investigators. All of the results to date, so far as known, have been completely negative. It would be a very major accomplishment, and a tremendous forward step, if we had some type of biologic test. At present nearly all knowledge of this disease is based on studies in human volunteers, carried on by courageous investigators and subjects.

A second area of deficiency, from a purely practical clinical standpoint, is the lack of a nonoperative means of visualizing the biliary duct system in the jaundiced patient. As you well know, the means of arriving at a clinical diagnosis is often devious and based on roundabout methods of approach. The methods include the customary history, the physical examination, and employment of nonspecific laboratory tests. Occasionally a more direct approach, that of needle biopsy of the liver, is made. At best, all of these are relatively unsatisfactory in the attempt to arrive at an anatomic explanation for the presence of jaundice in a given patient Thus

the availability of a roentgenologic method that would permit visualization of the extrahepatic bile duct system would be extremely advantageous

A third area of deficiency lies in our lack of understanding of the basic mechanisms responsible for the cirrhotic diseases The terminology, "cirrhotic disease," was used recently in a paper by Bradley and his associates (76). I think it is a good term inasmuch as it does not commit us to a single entity

There is an urgent need to reproduce, in the experimental laboratory, a counterpart of what may best be termed "postnecrotic cirrhosis." I believe that we have an experimental counterpart of the form of human cirrhosis that occurs in the chronic alcoholic, that patient who is a victim of "selective starvation," i e., a deficiency of lipotropic factors relative to his total caloric intake The similarity between cirrhosis as it occurs in the alcoholic patient, and the diffuse fibrosis or fatty cirrhosis as it is produced in the laboratory, is, it seems to me, too striking to deny I say this both from the functional and the anatomic standpoint

However, we cannot produce, at least so far as I know, an anatomic derangement in animals that simulates the postnecrotic type of cirrhotic disease so frequently seen among patients Such patients are not the victims of a recognizable deficiency disorder, and there seems little reason to believe that inadequate nutrition plays a role in the inception of that type of cirrhosis

We perhaps make a mistake when we tend to regard all cirrhosis occurring in nonalcoholics as examples of chronic viral hepatitis I think that may be deceptive It would be much better to admit, as I am sure most of us do, that the disease is idiopathic It is difficult to escape the conclusion that some cases of postnecrotic cirrhosis may be the result of viral hepatitis

I have attempted to produce postnecrotic cirrhosis in the rat Some of the studies have been reported (77,78), and are known to most of this audience The results are negative, recurrent attacks of dietary necrosis in the rat lead to postnecrotic scarring, but not to cirrhosis

Best. I invite other clinicians to supplement this introduction Dr Watson

Watson There are many things that come to mind Yesterday at a meeting in Philadelphia as I listened to a dissertation on the homeostasis of iron, my thoughts turned to the problem of hemochromatosis In the more recent literature, there has been considerable interest in so-called exogenous hemochromatosis I think there

is no doubt that there is a form of hemochromatosis in which there is an enormous storage of iron in the liver, with fibrosis of the liver and of the pancreas, as a result of chronic anemia. Such individuals have often received iron for long periods of time and many blood transfusions. It seems to be rather clear that the amount of iron in the livers of these individuals is often greatly in excess of what they received in the transfusions.

However, the interesting question is, why does fibrosis of the liver occur? Does the iron itself cause the fibrosis? That is a question that I think the experimentalists could give more thought to and try to delineate. Of course, it is true that hemochromatosis is uncommon and therefore relatively unimportant; nevertheless the whole question of iron storage is extremely interesting from other points of view, and I think it deserves more study than it has received.

Best: Did the Gillmans produce experimental hemochromatosis?

Gyorgy: No; Walker (79) in South Africa used the native Bantu diet, consisting of corn meal, with a high iron content, and was able to produce siderosis. One should not call it hemosiderosis, but siderosis.

Watson: Finch and his co-workers (80,81) have studied this problem of iron deposition in the livers of experimental animals I think, Dr Hoffbauer, you have some information on this subject.

Hoffbauer: Before I comment, I should like to draw Dr. Bollman out on this subject; I do not believe he has ever published the results of some of his studies that go back for quite a time.

Bollman: Dr. Hoffbauer is referring to the fact that a number of years ago I gave dogs intravenous hemoglobin and iron injections and produced hemosiderosis The livers of these animals contained tremendous amounts of iron After a few months of survival following the repeated iron injections, liver biopsies were taken, and at that time I suspected that early lesions of cirrhosis were developing However, these lesions were not definite and did not progress in animals which have survived for as long as ten years, over which time large amounts of iron remained in the liver and spleen I do not believe that the mere presence of iron in the liver is a determining factor in the production of the cirrhosis

Best It seems to me very interesting that you could maintain a very early cirrhosis

Bollman I do not believe I could call the lesions that I saw early cirrhosis I am sure now that these changes were not precirrhotic, but more likely minor changes in the liver organization as a result

of the presence of large amounts of iron I am quite satisfied that in the dog at least, large amounts of iron can remain in the liver without producing any functional or progressive change in the liver structure Actually the iron deposits in the liver appear as small inclusions of rust, but seem to be entirely inert Hemochromatosis does not develop.

Gyorgy: Iron is still in the liver, and in the same amount?

Bollman· Probably less, but still from 20 to 30 times more than normal

Best: That must mean new cells have taken up the iron, it could not be the same old cells.

Bollman The iron going into the liver is transferred You see it accumulating first in the Kupffer cells, and then these cells seem to disintegrate, after that the iron goes into the hepatic cells, and then you may see what seems to be the same iron back again in the Kupffer cells at a later stage Thus, the iron is not fixed, it is floating in the changing cells

Lillie Have you ever tried giving any radio iron to some of these animals that are already loaded with iron, to see if there is any interchange?

Bollman No, I have not I rather imagine that there would be an interchange, but I have not had that in mind

Shorr. Does anyone know what is the state of the ferritin in these livers? Is it fully saturated with iron? Why does this particular iron escape the usual fate of iron in being stored in the usual depots for hemoglobin formation?

Hoffbauer I cannot answer that question, but I should like to comment on the subject of iron deposition as influenced by restricted diets Hegsted, Finch, and Kinney (80,81) reported several years ago that enhanced absorption of iron occurred when animals were fed a diet low in phosphate One can certainly obtain an enhanced absorption of iron by such means The liver is very heavily infiltrated with iron, and the deposits are much as Dr Bollman has described Initially the iron accumulates about the portal areas in the rat The studies of Hegsted, Finch and Kinney were of rather brief duration Their animals rapidly lost weight, few of them survived over 60 days

I have attempted to improve the diet without disturbing its low phosphate content In order to prevent other factors which might influence changes in the hepatic cells, the diets were augmented with lipotropic agents We were able to carry animals as long as six months Most of the rats died of a massive necrosis of the liver.

but I did not recognize this lesion initially. Because of the dark mahogany color of the liver, the usual gross pathologic picture of massive dietary necrosis was obscured. We have restarted the experiments, and have added tocopherol in an attempt to prevent that disaster. So far as I have been able to tell in these various studies, there is no evidence that the iron *per se* damages the hepatic parenchyma, or stimulates fibrosis.

We have subjected a small series of animals to carbon tetrachloride inhalation to see what sudden necrosis of liver cells, and the consequent liberation of iron, or iron-containing pigment, might do. Would this prove more disastrous than a similar episode of necrosis in an animal whose liver was not loaded with iron? In preliminary experiments we have seen no difference in the behavior or survival of two groups of rats, one with iron-laden livers, and the other with normal livers, both developing a comparable degree of necrosis after carbon tetrachloride exposure.

Lillie: The iron that we see in hemochromatosis is commonly described as a hemosiderin. I should like to ask if we know anything about the relationship of ferritin, or apoferritin, to the protein matrix which is known to exist in hemosiderin.

Shorr: I can only tell you what we know about the iron of ferritin. We know that ferritin can incorporate variable amounts of iron up to 24 per cent, and that the state of the iron is related to the state of oxidation or reduction of the sulfur groups in ferritin. In the oxidized disulfide state, the iron is present largely in the ferric form and is tightly bound to the ferritin molecule. When ferritin is reduced to sulfhydryl ferritin, there is an increase in the Fe^{++} ions which are more labile. This iron is readily removed from ferritin *in vitro* by the iron-binding globulin. Our studies with Dr. Mazur (82) have also shown that the state of oxidation or reduction of ferritin in the body alters the ease in which iron is released from ferritin. When the liver becomes hypoxic, as in shock, the ferritin is reduced to the sulfhydryl state, and an increased number of ferrous irons is reflected in changes in the blood stream. There is an increase in plasma iron and in the saturation of the iron-binding globulin. These experiments would suggest that the movement of iron in and out of ferritin may be achieved by its oxidation or reduction. Once ferritin is reduced and more Fe^{++} iron formed, then the circulating iron-binding globulin will pick up and oxidize it to the ferric form, protecting the organism from the potential vasodepressor actions of iron, and carrying iron to sites of hemoglobin formation.

Lillie: The iron content of hemosiderin may run higher.

Shorr: How high does it go?

Lillie: I think there are figures that suggest from 70 to 80 per cent, or perhaps even higher.

Watson: Dr. Lillie, has it been established that hemosiderin is a true complex with a protein?

Lillie: There are indications that that is so. We know that we can remove the iron from hemosiderin and leave a material behind which gives the periodic acid Schiff, or other reactions, which are given by some hemosiderins. Pulmonary heart failure cells give a quite sharp hemosiderin reaction. The iron may be removed from that pigment by oxalic or sulfuric acid. The pigment itself, with its periodic acid Schiff reaction, remains clearly visible.

Shorr: The use of antiferritin serum might be helpful in resolving this problem.

Lillie: Probably our best source of copious hemosiderin is the lung of the chronic heart failure case.

Goldblatt: So far, all those who have spoken about hemochromatosis have mentioned only the iron-containing pigment, and have made no reference to the other material, hemofuscin, which is supposed to accompany the deposit of iron-containing pigment in this condition. It is not present when there is hemosiderosis on any other basis, such as cardiac failure, pernicious anemia, or siderosis from any cause, such as inhalation of metallic iron.

Lillie: That has been disputed, has it not?

Goldblatt: Yes, I was wondering whether this had not been disproved completely, and whether this other material, rather than the iron-containing pigment, was not the fibrosis-producing substance. When we have a long-standing secondary anemia, for example, or a primary anemia, there is profound hemosiderosis in the pancreas and other organs, yet there is no fibrosis. We do see fibrosis in the lung, from the inhalation of metallic iron. Thus, the form of the iron may be the determining factor.

Lillie: Von Recklinghausen's (83) hemofuscin appears to have been distributed largely in smooth muscle rather than in liver, and as nearly as I can discern from the literature, there is not very much to distinguish it from the brown pigment that one finds in smooth muscle of the intestinal tract under noniron-bearing pigmentation conditions. I believe Werner Hueck (84,85) actually considers the hemofuscin, so-called, as just another one of the lipofuscin pigments.

Best: Dr. Popper, would you like to present your material?

Popper: I should like to refer as briefly as I can, to the results of the administration of ethionine to rats, and omit here the discus-

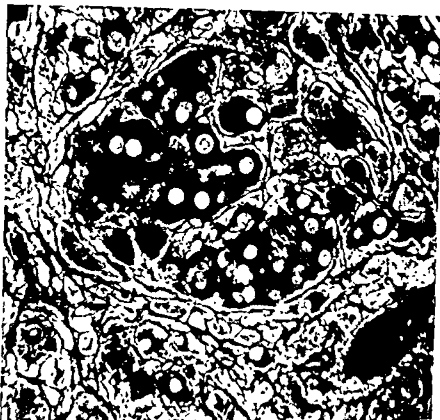


FIGURE 45 Liver of rat on 0.5 per cent ethionine diet for 70 days Small nodules composed of liver cells arranged in plates several cells thick, differing from the surrounding parenchyma which reveals extensive interstitial fibrosis Gomori silver impregnation. (x350).

What has interested us most during the past few months have been findings in rats on the ethionine diet for from 70 to 80 days, and which were then returned for almost a year to a normal diet. In these rats a peculiar type of septal cirrhosis develops with septa dissecting the parenchyma in an irregular fashion In addition, large nodules are found (Figure 49). They consist mainly of dense connective tissue and a few bile duct proliferations which appear to be choked off by the connective tissue (Figure 50). However, one year after the discontinuation of the ethionine diet, active proliferation and mitoses (Figure 51) are noted, in addition to mucus production which these ducts share with the proliferates in acute cholangiofibrosis, but which normal bile ducts fail to show. We cannot prove today that the lesion produced represents a neoplasm,

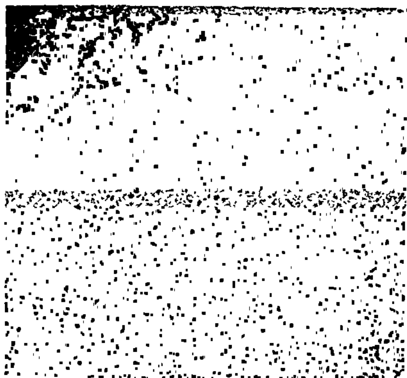


FIGURE 46 Liver of rat on 0 5 per cent ethionine diet for 51 days Sharply demarcated nodule composed of liver cells which are large and arranged in plates several cells thick Outside the nodule there is severe interstitial fibrosis H&E stain (x60).

but we are exceedingly interested in this autonomous behavior of the regenerates which maintain their activity even a year after the insult has been removed

One of the reasons for our interests in this lesion was the one which Dr Hoffbauer brought up, namely, the attempt to produce experimentally a lesion similar to ones found in clinical medicine I wish to give credit to Dr Davies (90), whose discussions of kwashiorkor before this group stimulated my personal interest in the lesions produced by ethionine

In conclusion, I should like to say that it was possible to produce with ethionine· fatty liver, diffuse hepatocellular damage with fibrosis, cirrhosis, and finally, hepatic lesions on the border of tumor formation We have not yet been able to produce an unquestionable



FIGURE 47. Liver of rats on ethionine diet, H&E stain. 0.5 per cent diet for 51 days. Irregular proliferation of small bile ducts near portal triads with mitosis and mucus production (x215).

FIGURE 48. 0.2 per cent diet for 104 days. Subcapsular nodule consisting of proliferated mucus producing bile ducts and connective tissue (x60).

carcinoma with metastases. Finally, as reported here before, pancreatic degeneration occurs, proceeding to a diffuse pancreatic atrophy and fibrosis, again reminiscent of kwashiorkor. Moreover, in some rats we found glycosuria and an abnormal blood sugar tolerance curve.

Hartroft: Do pancreatic tumors appear in these rats?

Popper: We obtain very bizarre regeneration of the pancreatic cells, but not tumors.

Neefe. Does jaundice develop?

Popper. Some of the rats excrete bilirubin in the urine. In the acute stage the serum bilirubin may rise to about 3 mg. per cent. Obvious jaundice is not noted.

Houssay. What is the diet of these animals?

Popper. It consists of 16 per cent vitamin-free casein, 5 per cent corn oil, 75 per cent sucrose, and 4 per cent salt mixture and vitamin supplements, the riboflavin content is on the border line, being 0.42 mg. per 100 gm. of the diet. We can increase the protein con-

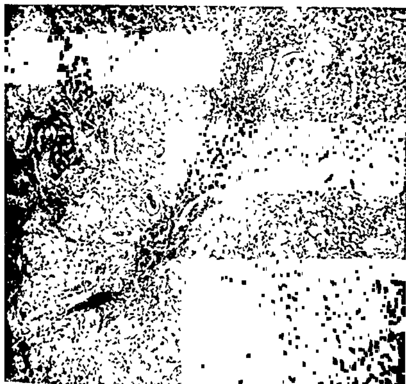


FIGURE 49 Liver of rat alternating on 0 5 per cent ethionine and normal diet for 170 days, and subsequently for one year on stock diet Dissection of lobular pattern by septa, and large nodules consisting of sclerosing connective tissue with few bile duct proliferates H&E stain (x60)

tent and still produce the lesion, if we simultaneously increase the ethionine supplements

Lillie. Do these large clear cells store glycogen?

Popper Some of the cells in the nodules are rather rich in glycogen, in contrast to the surrounding tissue

Best: How much ethionine is in the diet?

Popper: It contains 0 5 per cent ethionine, and the methionine content of the protein of the diet is around 0 4 per cent As mentioned, we can raise the protein content if we simultaneously raise the ethionine concentration

Watson: Does choline provide a protection?

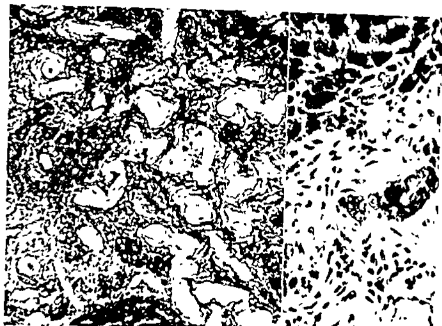


FIGURE 50 (Same rat as Figure 49) Marked proliferation of connective tissue in form of membranes choking off few proliferating bile ducts Mallory stain (x150)
FIGURE 51 Isolated bile duct proliferates in sclerosing connective tissue revealing mitoses and mucus production H&E stain (x320).

Popper: Choline gives no protection from the acute fatty liver developing within 48 hours in female rats. In contrast, raising the choline content of the synthetic ethionine diet, administered for five weeks, produces a peculiar lesion in the chronic experiments (Figure 52). The ethionine lesion is markedly attenuated and some liver cells show regeneration, whereas others are a bit damaged Fissures may be seen within the parenchyma, and the liver cells contain, peculiarly enough, some fat vacuoles in contrast to animals on ethionine alone without the choline supplements.

Watson Why do you think you get a fatty liver when you give choline?

Popper. It is not really a fatty liver. The fat content is higher than normal; that is, approximately 9 per cent, whereas in the ethionine-treated rats without choline it is about 3 per cent.

Watson Why do you get anything at all when you give choline?

Popper I cannot explain that, and it brings up a rather complicated question Stekol (89) has recently shown that the basic metabolic defect in ethionine intoxication is related to a defect in

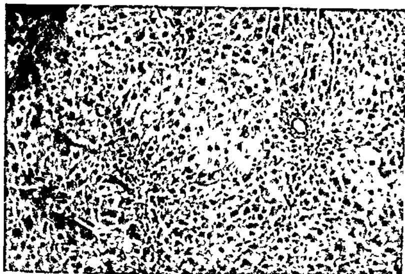


FIGURE 52 Rats on 0 5 per cent ethionine diet for 30 days supplemented by 0 6 per cent choline Irregular patchy regeneration of liver cells with fine vacuolization Stress fissures traversing the lobular parenchyma Little inflammatory reaction is noted in the parenchyma H&E stain (x120)

the choline metabolism Nevertheless, choline does not give full protection in ethionine intoxication In experimental protein, malnutrition choline does not prevent some types of liver cell damage (91)

Lillie This is excessive choline, of course, Dr Popper

Popper Not very excessive The choline concentration in the supplemented diet was 0 6 per cent, in contrast to the nonsupplemented diet in which it was 0 03 per cent

Goldblatt Even if fat is not clearly seen in H&E stains, we can demonstrate it with fat stains How does the quantity compare with a liver which is the seat of pronounced fat infiltration?

Popper The normal liver has a fat content of 5 gm per 100 gm wet tissue In chronic ethionine intoxication, it goes down as low as 3 per cent

Goldblatt Give me the figure for the obviously fatty liver

Popper Fifteen per cent

Goldblatt You mean the advanced fatty change? Ten per cent of wet weight is of course above normal, but 15 per cent represents fully developed fatty metamorphosis

Best: It goes up to 60 per cent. It seems to me there is perhaps some sort of conflict between the toxic effect of ethionine, and a lack of lipotropic factors; that is, when the two things work together. The explanation of the increased fat, when choline is given, must lie along that line, I think.

Popper: The administration of choline may restore certain metabolic function.

Best: That is what I mean.

Popper: Whether this would indicate that ethionine produces a toxic effect independent of the antagonism to methionine, I do not know.

Best: Are there any other indications of the toxicity of ethionine?

Popper: So far, we have studied only the lesions in the liver and pancreas. They have been prevented by the simultaneous administration of methionine, and have very rapidly been corrected if methionine were given, or if the animals were put on a normal diet. Even the severe pancreatic lesion is quickly corrected. We have recently learned about cardiac alterations due to ethionine but we have not yet reviewed our material for such lesions The described lesions are not due to triethylcholine intoxication (a possible metabolite of choline) since feeding of this substance does not produce them. We have so far not found any evidence for toxicity.

Goldblatt: Perhaps Dr. Popper will dispute this with me, but I am inclined to agree with Dr. Best that this is not in any way a choline-deficient liver. We are not looking at a liver about which we can say, objectively, "This is fat infiltration." It is obviously not At most, one would say fatty degeneration, presumably as a result of the toxic effect that Dr. Best is speaking about. I am just wondering whether we can speak of the fat content of a liver, as a whole, and compare one with another in that respect We look at that liver, and may not even think of its being fatty at all. Under high power, we see tiny vacuoles that we call fatty degeneration. You say you *have given choline and there is no deficiency* We grant you that, that picture is not the result of a deficiency. It is probably so-called fat phanerosis, rather than fat infiltration.

Popper: That is possible. I should like to emphasize again that within the first few days of the feeding of ethionine, a severe fatty liver develops which contains chemically more than 15 per cent fat. Subsequently, the fat disappears from the liver and histologically none is seen at all, whereas chemically the fat content is considerably below that of normal rats. If liberal choline supplements are

given with prolonged ethionine diets, the fat content is not below but slightly above normal, and small amounts of fat can be seen histologically. This was the picture to which I referred last

Hartroft: I wonder whether Dr Popper would comment on the intensely eosinophilic liver cells in Figure 49 illustrating the field containing trabeculae? What distribution do they possess, in relation to the radicles of the hepatic vein and the branches of the portal vein? Does the addition of choline to a diet containing ethionine modify the development of the pancreatic lesions in any way?

Popper: The markedly eosinophilic liver cells indicate degeneration or necrobiosis. They are not necrotic yet This acidophilic degeneration of the cytoplasm is especially clearly seen in Mallory or trichrome stain It is not only due to loss of cytoplasmic basophilia, but also to a degenerative change of the cytoplasm

Neefe: Is it somewhat similar to the cells you see in acute hepatitis?

Popper: No In viral hepatitis, diffuse acidophilic degeneration is associated with pycnosis of the nuclei In these instances the nuclei are vesicular, but probably the process is of a similar nature

The distribution of these septa has interested us very much, but we have not yet made any reconstructions They appear to be neither central nor portal, but extend straight through the parenchyma without any obvious relation to the lobular topography Dr M. Hans Elias and I believe that they result from stress fissures between regenerating and nonregenerating territories of the lobular parenchyma into which connective tissue membranes are laid down

Hartroft. I wonder whether the distribution of these trabeculae might not be related to the structural units of the liver described by Rappaport recently (92). The figures which have been shown suggest to me that the trabeculae might be found to outline Rappaport's structural units

What influence, if any, do dietary choline supplements have on the development of ethionine-induced lesions of the pancreas?

Popper. The pancreatic lesion is not prevented by liberal choline supplements to the ethionine diet, but so far we have the impression that it may be milder

Shorr: Are there any kidney changes?

Popper: The renal changes are nonspecific degenerative alterations of the proximal convoluted tubules, and protein is found in the urine

Best: Dr. Hanger, do you have some ideas about what the experimentalists should do to make that picture in the liver clearer?

Hanger: That is an appropriate question to ask at the end of our series of conferences. I am afraid there are a great many things yet to be learned. For example, it is regrettable that liver function tests, which are really measurements of physiological activities, are still symbols rather than indices of known integrated activities Many of them, especially those determining the levels of metabolites in the blood, represent equilibria which are disturbed in certain hepatic derangements and are therefore of clinical value, but the anabolic, catabolic and excretory factors, which regulate these levels, are mostly unknown. Some of the excellent chemical studies reported in these conferences help us to comprehend the complexities of the problem, and certainly remind us of our lack of understanding of what we are really measuring when we perform the simplest liver function test, such as the serum alkaline phosphatase determination, or the total serum cholesterol

Dr. Hoffbauer brought up the question of how we can explain chronic progressive hepatic injury I think, too, that it is time to challenge the idea that all cases of chronic hepatitis are due to chronic viral infections. There are classical examples of chronic inflammatory changes in joints, in the kidneys, indeed in every organ of the body, which are not due to infection

As to the causes of liver injury, I think most of them are still unknown. I believe that Dr. Popper's presentation is just an example of noninfectious chronic degeneration. Whether the causes are dietary in most cases, I doubt. Perhaps we may learn from dermatologists and students in other fields about these chronic inflammatory diseases.

Watson: Are you subscribing to the idea that chronic ideopathic cirrhosis is just a form of lupus erythematosus

Hanger· If I knew what these conditions represent, I could answer that question.

Watson· It sounded that way when you said the dermatologists were going to help us

Hanger The pathologists also might help The question of the pathology of hepatitis is still a baffling one Most viruses are very specific as to the tissues that they injure; yet in chronic hepatitis we find a tremendous reaction in the mesenchymal elements of the liver as well as in the parenchyma, which are really two entirely different structures. I think the question is still far from answered, whether parenchymal damage leads to secondary mesenchymal

injury, or whether there is a virus that happens to involve both tissues It is intriguing that in the story of hepatitis we frequently see skin reaction, joint pains, lymph gland enlargement, and enlargement of the spleen, suggesting that in addition to viral infection there may be a hypersensitive state to the virus, giving reactions similar to those found in serum disease Thus, the question comes, whether we do not have both a parasitism on the part of the virus, and also hyperimmune reactions to modify the clinical picture, or actually precipitate the acute attack

Best· Dr Neefe, do you sometimes give cortisone to people with failing liver?

Neefe. Yes, I think a good many people do now, Dr Best Dr Hanger has just mentioned what I have long suspected is an important factor in certain liver diseases namely, the question of hypersensitivity. I think anyone familiar with hepatitis has been impressed with the long incubation period as compared with other diseases, and we have all been impressed with the frequency of urticaria, and other general phenomena, which are often among the premonitory symptoms and signs

I should be very much interested in seeing what could be done with the phenomenon of hypersensitivity, induced in experimental animals on a chronic basis with one or another agent I think it might be a very fertile field Perhaps we might add to that certain other known toxic agents

Some of us have also been impressed with the relative frequency of associated conditions in people who have chronic liver disease with associated latent amebiasis Dr Richard Capps, and others, have been impressed with the frequency of such conditions as intestinal amebiasis in such patients Certainly I have observed with a great deal of interest the favorable effects of ACTH or cortisone in some cases of chronic hepatitis, very dramatic responses occurring occasionally which would be difficult to explain except perhaps on the basis of interruption of a hypersensitivity process

Like Dr. Hanger, I have tried to think of other puzzling problems in clinical liver disease For example, I have seen a number of patients with chronic or subacute liver disease who have duodenal ulcer. It has often been stated that it is quite common, in chronic liver disease, to find associated ulcer Perhaps we should consider it in the reverse direction If one goes into the history of such patients, one may find symptoms suggestive of an ulcer long antedating the apparent liver disease One might speculate as to whether

there might be absorption of sensitizing products through an ulcer resulting in sensitization of susceptible persons. There are many factors of this sort which may eventually be shown to contribute to these complex chronic diseases.

Best: Dr. Shorr, would you now speak as a clinician?

Shorr: I should like to learn more about the relation of the liver to vascular homeostasis. We have already learned a good deal about the effect of certain conditions of the liver on the ability of experimental animals to respond to the stress of hemorrhagic and traumatic shock. Impaired protein nutrition was shown by Dr. György to jeopardize the survival of rats to even minor surgical procedures. It was from his observations that we were stimulated to pursue this line of research in shock. We should also like to know more about the relation of the liver to the whole problem of oliguria, which we encounter in various clinical disturbances such as decompensated cirrhosis, nephrosis, and heart failure with edema These conditions are regularly accompanied by ferritinemia, and we have shown that ferritin is a powerful antidiuretic because of its stimulation of the neurohypophysis to release antidiuretic material We would like to know much more about the role of ferritinemia in those clinical oliguric states to the impairment of water excretion.

Best: Professor Hoet, what do you think the experimentalists should do to help the clinicians?

Hoet: I was thinking of asking if someone knows whether jaundice during pregnancy usually leads to abortion. Hench (93) has observed an increase of glucocorticoids. In case of jaundice there may be more, and then we might expect that there would be abortion It is very difficult to state this. The cases are complex and it is not a pure question of glucosteroids. However, I should still like to ask the question.

Watson: I shall be glad to answer it, at least on the basis of my own experience. I think I have seen perhaps eight or ten cases of hepatitis in pregnancy, and the patients have done quite well, for the most part A paper has recently been published on this question by Martini (94). He described some fifty cases of infectious hepatitis associated with pregnancy. He had a large enough series to compare them with hepatitis without pregnancy, and no significant difference could be found I have seen a number of cases of common duct stone with jaundice in pregnant women without creating a situation more adverse than in ordinary cases. We have had a few of these women operated on because they were having chills and fever, but this has usually not terminated the pregnancy, in my experience.

Neefe: I think perhaps the stage of pregnancy in which the disease occurs might be very important. My experience has been much the same as that of Dr Watson, although most of those I have seen have been in the first trimester of pregnancy. They have all done well Yet I have heard of another series in late pregnancy, and many of those patients did abort, or had premature delivery

Hoet. There was a very bad epidemic reported by Dr. H Staub (95). The pregnant women had more and heavier jaundice than the others in the population In that situation, they aborted or even died However, that does not indicate that it was glucosteroids, it was a particular type of virus, I suppose, that existed at that time in the town of Basle I think the reaction of the patient will depend upon the cause of the jaundice, and on the stage of pregnancy at which the disease is contracted However, in any case, it is not very plain If they abort, it is not very easy to state the phenomenon which caused it

Watson. That virus in Basle was a most unusual one Staub (96) stated that the mortality ran up to about 20 per cent, at least for a period of time.

Best: Do you have any comments, Dr Gyorgy, either as a clinician or an experimentalist

Gyorgy· Our major defect in experimental work is that we cannot reproduce kwashiorkor in rats by dietary means If it were protein deficiency alone, we could certainly use protein-deficient diets, but in rats fed rations which are low in casein the result is fatty liver, there is cirrhosis, but not kwashiorkor as seen in human infants Engel and his associates (97) were at least successful in producing fatty liver, cirrhosis and generalized edema in rats fed a diet containing peanut meal and small amounts of casein That is more suggestive of kwashiorkor than uncomplicated liver injury in rats fed rations containing only casein With ethionine, Dr Popper was able to produce changes in the liver and in the pancreas Pancreatic changes were not found by us in rats kept on a diet containing only casein Finally, in rats with dietary fatty liver the fat infiltration is central, in kwashiorkor it starts in the periphery

Best Shall we agree that there are still many fascinating clinical and experimental problems?

REFERENCES

1 VENDRELY, R, and VENDRELY C La teneur du noyau cellulaire en acide desoxyribonucleique a travers les organes, les individus et les espèces animales *Experientia* 4, 434 (1948)

2. Campbell, R. M, and Kosterlitz, H W.: The absence of dietary effects on the DNA content of liver nuclei of the adult rat. *Science* 115, *84 (1952).*
3 Thomson, R. Y., Heagy, F. C, Hutchison, W. C, and Davidson, J N · *The deoxyribonucleic acid content of the rat* cell nucleus and *its* use *in* expressing the results of tissue analysis, with particular reference to the composition of liver tissue. *Biochem. J* 53, *461 (1953).*
4. Davidson, J N : Les nucléoprotéines et la croissance des tissues *Bull. Soc. chim. biol.* 35, *49 (1953).*
5. Campbell, R. M, and Kosterlitz, H. W.: *The effects* of growth and sex on the composition of the liver cells of the rat *J Endocrinol. 6, 308 (1950).*
6 ———: The effects of dietary protein, fat and choline on the composition of the liver cell and the turnover of phospholipin and protein-bound phosphorus. *Biochim. et biophys acta* 8, *664 (1952).*
7. Ely, J. O, and Ross, M. H · Desoxyribonucleic acid content of rat liver [illegible]
8 Popper, H.· [illegible] on liver biops[illegible] Trans Ninth Conf New York, Josiah Macy, Jr Foundation, 1951 (p. 9)
9 Bendich, A, Russel, P. J, Jr., and Brown, G. B · On the heterogeneity of the desoxyribonucleic acids *J Biol Chem* 203, *305 (1953).*
10 Billing, B H, Conlon, H J, Hein, D. E, and Schiff, L.· The value of needle biopsy in the chemical estimation of liver lipids in man. *J. Clin. Investigation* 32, *214 (1953).*
11. Popper, H, and Wozasek, O.· Zur Kenntnis des Glykogengehaltes der Leichenleber (Untersuchungen bei Diabetes mellitus) *Ztschr. f. d ges exper Med.* 77, *414 (1931).*
12 Popper, H. Distribution of vitamin A in tissue as visualized by fluorescence microscopy *Physiol Rev* 24, *205 (1944)*
13 Gillman, J., and Gillman, T.· *Perspectives in Human Malnutrition. A Contribution to the Biology of Disease from a Clinical and Pathological Study of Chronic Malnutrition and Pellagra in the African* New York, Grune, 1951
14 Stowell, R. E. Nucleic acids and cytologic changes in regenerating rat liver *Arch Path 46, 164 (1948)*
15 Yokoyama, H. O, Wilson, M. E, Tsuboi, K K., and Stowell, R. E Regeneration of mouse liver after partial hepatectomy. *Cancer Res* 12, *80 (1952).*
16 Brues, A M., Drury, D R., and Brues, M C · A quantitative study of cell growth in regenerating liver *Arch Path* 22, *658 (1936).*
17 Mendel, B The action of ferricyanide on tumour cells *Am J Cancer 20, 549 (1937)*
18 Mendel, B, and Strelitz, F Specific action of ferricyanide on aerobic glycolysis of tumour cells *Nature* 140, *771 (1937)*

19 ROSENTHAL, O, ROGERS, C. S, VARS, H. M, and FERGUSON, C. C., Arginase, adenosinepyrophosphatase, and rhodanese levels in the liver of rats *J. Biol Chem* 185, 669 *(1950)*
20 WATERLOW, J: Enzyme activity in human liver *Liver Injury* F. W. Hoffbauer, Editor Trans Eleventh Conf New York, Josiah Macy, Jr Foundation, 1953 (p 72)
21 ROURKE, G M., and STEWART, J D Composition of the liver, its uniformity with respect to the concentration of certain biochemical constituents in different parts of the same liver *Arch Path* 33, 603 *(1942)*
22 THEIS, E R. The lipid distribution in normal and abnormal liver tissues; the effect of disease upon the lipid distribution in human liver tissue *J Biol Chem* 82, 327 *(1929)*
23 WITH, T. K.: Micro-method for determination of vitamin A in liver biopsies in man and larger animals *Biochem J* 40, 249 *(1946)*.
24 GOMORI, G, and GOLDNER, M G Uneven distribution of glycogen in the liver *Proc Soc Exper Biol & Med* 66, 163 *(1947)*
25 CHAIKOFF, I L, and KAPLAN, A Distribution of fat in the livers of depancreatized dogs maintained with insulin *J Biol Chem* 119, 423 *(1937)*
26 HENRY, K. M, KOSTERLITZ, H W, and QUENOUILLE, M H A method for determining the nutritive value of a protein by its effect on liver protein *Brit J Nutrition* 7, 51 *(1953)*
27. BLOCK, R J, and MITCHELL, H H The correlation of the amino-acid composition of proteins with their nutritive value *Nutrition Abstr & Rev* 16, 249 *(1946 1947)*
28 CANNON, P R · The problem of tissue protein synthesis *Federation Proc* 7, 391 *(1948)*
29 STEFFEE, C H, WISSLER, R W, HUMPHREYS E M BENDITT, E P, WOOLRIDGE, R W, and CANNON P R Studies in amino acid utilization V The determination of minimum daily essential amino acid requirements in protein depleted adult male albino rats *J Nutrition* 40, 483 *(1950)*
30 VARS, H M, and GURD, F N Effect of dietary protein upon the regeneration of liver protein in the rat *Am J Physiol* 151, 399 *(1947)*
31. CAMPBELL, R M, and KOSTERLITZ, H W The assay of the nutritive value of protein by its effect on liver cytoplasm *J Physiol.* 107, 383 *(1948)*
32 ROGERS, C S, FERGUSON, C C, FRIEDGOOD C F and VARS, H M Influence of fat in the diet upon nitrogen balance and liver regeneration *Am J Physiol* 163, 347 *(1950)*
33 ELMAN, R, DAVEY, H W, and KIYASU, R Nitrogen balance on a restricted caloric intake *J Lab & Clin Med* 30, 273 *(1945)*
34 *Technical Report Series No 72 Joint FAO/WHO Expert Committee on Nutrition, Third Report* World Health Organization, Geneva, 1953

35. MUNRO, H. N.. Carbohydrate and fat as factors in protein utilization and metabolism *Physiol. Rev.* 31, *449 (1951)*
36. BEST, C. H, HARTROFT, W. S, LUCAS, C. C, and RIDOUT, J H Liver damage produced by feeding alcohol or sugar and its prevention by choline. *Brit. M. J.* 2, *1001 (1949).*
37. KLATSKIN, G, GEWIN, H M, and KREHL, W. A. Effects of prolonged alcohol ingestion on the liver of the rat under conditions of controlled adequate dietary intake. *Yale J. Biol. & Med.* 23, *317 (1951).*
38 FISHMAN, W H, and ARTOM, C · The relation of the diet to the composition of tissue phospholipides; liver lecithin as related to the choline and fat content of the diet *J Biol. Chem* 164, *307 (1946).*
39 DAVIDSON, J N, and LESLIE, I: *A new approach in the biochemistry of growth and development* *Nature* 165, *49 (1950).*
40 KOSTERLITZ, H. W: The effects of changes in dietary protein in the composition and structure of the liver cell *J Physiol* 106, *194 (1947).*
41. CAMPBELL, R. M, and KOSTERLITZ, H. W: The effect of dietary protein on the turnover of phospholipides, ribonucleic *acid, and desoxyribonucleic acid in the liver J. Biol Chem* 175, *989 (1948)*
42 MUNRO, H N, NAISMITH, D. J, and WIKRAMANAYAKE, T. W: The influence of energy intake on ribonucleic acid metabolism *Biochem J* 54, *198 (1953).*
43 FLOCK, E V, BOLLMAN, J L, and BERKSON, J · Effect of thyroxine and thiouracil on the rate of phospholipid turnover *in the liver of the rat. Am J Physiol* 155, *402 (1948)*
44 CAMPBELL, R M, and KOSTERLITZ, H. W.. Some effects of pregnancy and lactation on the liver. *J. Endocrinol.* 6, 171 *(1949)*
45 ADDIS, T, POO, L. J, and LEW, W Protein loss from the liver during a 2 day fast *J. Biol Chem.* 115, *117 (1936)*
46 CAMPBELL, R M, and KOSTERLITZ, H. W.: The influence of sex on liver cytoplasm *J Physiol* 105, *33P (1946).* (Cambridge Univ. Press)
47 ——— The relationship between losses in labile liver cytoplasm *and urinary nitrogen excretion Biochem. J* 43, *416 (1948)*
48 MILLER, L L · The loss and regeneration of rat liver enzymes related to diet protein *J. Biol Chem* 186, *253 (1950)*
49 LITWACK, G, WILLIAMS, J N, JR, FEIGELSON, P, and ELVEHJEM, C A Xanthine oxidase and liver nitrogen variation with dietary protein *J Biol Chem.* 187, *605 (1950).*
50 CAMPBELL, R M, and KOSTERLITZ, H W: The effects of *short term changes in dietary protein on* the response of the liver to carbon tetrachloride injury *Brit J. Exper. Path* 29, *149 (1948)*
51 ——— Protein and nucleic acid contents of rat livers one day after a single injection of carbon tetrachloride *ibid* 33, *518 (1952)*

52 SOLOMON, G, and TARVER, H The effect of diet on the rate of loss of labeled amino acid from tissue proteins *J Biol Chem* 195, *447 (1952)*
53 CLARK, I. The effect of cortisone upon protein synthesis *J Biol Chem* 200, *69 (1953)*
54 SILBER, R H, and PORTER, C C Nitrogen balance, liver protein repletion and body composition of cortisone treated rats *Endocrinology* 52, *518 (1953)*
55 HARRIS, L. J, BLAND, M N, and HUGHES, R E Effect of ACTH and cortisone on vitamin C metabolism and on the weight and composition of the liver in guineapigs and other species *Lancet* 1, *1021 (1953)*
56 LUCK, J M Liver proteins, the question of protein storage *J Biol Chem* 115, *491 (1936)*
57 GOLDSCHMIDT, S, VARS, H M, and RAVDIN I S The influence of the foodstuffs upon the susceptibility of the liver to injury by chloroform, and the probable mechanism of their action *J Clin Investigation* 18, *277 (1939)*
58 SCHULTZ, J, and VARS, H M Hepatic purine nitrogen partition in normal and injured rats *Am J M Sc* 213, *632 (1947)*
59 MILLER, L. L, and WHIPPLE, G H Chloroform liver injury increases as protein stores decrease, studies in nitrogen metabolism in these dogs *Am J M Sc* 199, *204 (1940)*
60 MILLER, L. L. The loss and regeneration of rat liver enzymes related to diet protein *J Biol Chem* 186, *253 (1950)*
61 SCHULTZ, J On the nature of labile protein I The cathepsin II activity of the liver and kidney of the fed and fasted rabbit *J. Biol. Chem* 178, *451 (1949)*
62 MUNTWYLER, E, SEIFTER, S, and HARKNESS D M Some effects of restriction of dietary protein on the intracellular components of liver *J Biol Chem* 184, *181 (1950)*
63 WESTERFELD, W W, and RICHERT, D A Liver and intestinal xanthine oxidases in relation to diet *J Biol Chem* 192, *35 (1951)*
64 WILLIAMS, J N, JR, DENTON, A E, and ELVEHJEM, C A Effect of methionine deficiency upon enzyme activity in the rat *Proc Soc Exper Biol & Med* 72, *386 (1949)*
65 RICHERT, D A, and WESTERFELD W W Isolation and identification of the xanthine oxidase factor as molybdenum *J Biol Chem* 203, *915 (1953)*
66 POPPER, H, KOCH-WESER, D, and DE LA HUERGA, J Serum and hepatic enzymes in experimental liver damage *J Mt Sinai Hosp* 19, *256 (1952)*
67 ROSENTHAL, O, FAHL, J C, and VARS, H M Response of alkaline phosphatase of rat liver to protein depletion and inanition *J Biol Chem* 194, *299 (1952)*
68 ——— Qualitative and quantitative changes in alkaline phosphatase activity of regenerating rat liver *Am J Physiol* 171, *604 (1952)*

69. GURD, F. N, VARS, H. M, and RAVDIN, I S. Composition of the regenerating liver after partial hepatectomy in normal and protein-depleted rats *Am. J. Physiol.* 152, *11 (1948)*
70 CAMPBELL, R M, INNES, I R., and KOSTERLITZ, H W.· The role of hormonal and dietary factors in the formation of excess ribonucleic acid in the livers of pregnant rats *J. Endocrinol* 9, *52 (1953)*.
71 YEAKEL, E. H, and TOBIAS, G: Liver nitrogen in tumor bearing rats. *Cancer Res.* 11, *830 (1951)*.
72. ROSE, W. C.: The nutritive significance of the amino acids *Physiol. Rev.* 18, *109 (1938)*.
73 ——· Amino acid requirements of man *Federation Proc* 8, *546 (1949)*.
74. WHIPPLE, G. H, and MADDEN, S C.: Hemoglobin plasma protein and cell protein — their interchange and construction in emergencies *Medicine* 23, *215 (1944)*
75. COLBERT, J W., JR. Review of animal experimentation in infectious hepatitis and serum hepatitis. *Yale J Biol & Med.* 21, *335 (1949)*
76 BRADLEY, S E, SMYTHE, C. M, FITZPATRICK, H F, and BLAKEMORE, A. H: The effect of a portacaval shunt on estimated hepatic blood flow and oxygen uptake in cirrhosis *J Clin Investigation* 32, *526 (1953)*.
77 HOFFBAUER, F W., and WITTENBURG, B· Dietary hepatic necrosis in the rat absence of cirrhosis following recurrent episodes *Ann New York Acad Sc.* (In press)
78 —— Dietary hepatic necrosis· absence of cirrhosis following recurrent episodes *Federation Proc* 12, *392 (1953)*
79 WALKER, A R. P., and ARVIDSSON, U. B: Iron intake and haemochromatosis in Bantu. *Nature 166, 438 (1950)*
80. KINNEY, T D, HEGSTED, D M, and FINCH, C. A. The influence of diet on iron absorption I The pathology of iron excess *J Exper. Med* 90, *137 (1949)*.
81 HEGSTED, D M, FINCH, C. A, and KINNEY, T. D. The influence of diet on iron absorption II The interrelation of iron and phosphorus *ibid 147*
82 MAZUR, A, BAEZ, S, and SHORR, E· Relation of state of ferritin-iron to its biological activity *Federation Proc.* 13, *261 (1954)*.
83 V RECKLINGHAUSEN, F D: Ueber Hämochromatose. *Tagebl. 62 Versamml deutscher Naturforscher u Ärzte in Heidelberg* 62, *324 (1889)*
84 HUECK, W Die pathologische Pigmentierung *Handbuch der allgemeinen Pathologie* L. Krehl and F Marchand, Editors Vol III, No 2, chap 6, Leipzig, Hirzel, 1921 (p 298).
85 —— Pigmentstudien *Beitrage zur pathologischen Anatomie und zur allgemeinen Pathologie.* E Ziegler, Editor Vol 54, Jena, Fischer, 1912 (p 68)

86 FARBER, E, and POPPER, H The production of acute pancreatitis with ethionine and its prevention by methionine *Proc Soc Exper Biol & Med* 74, *838 (1950)*
87 POPPER, H · *Liver Injury* F W Hoffbauer, Editor Trans Eleventh Conf New York, Josiah Macy, Jr Foundation, 1953 (p 162)
88 KOCH-WESER, D, and POPPER H Hepatic fibrosis produced by chronic ethionine feeding *Proc Soc Exper Biol & Med* 79, *34 (1952)*
89 STEKOL, J A, WEISS, S, and PEN TUNG HSU Choline, creatine, and methionine synthesis in the rat in relation to ethionine metabolism *Abstracts, XIX Internat Physiol Congr Montreal, 1953*
90 DAVIES, J N P. Kwashiorkor *Liver Injury* F W Hoffbauer, Editor Trans Ninth Conf New York, Josiah Macy, Jr Foundation, 1951 (p 151)
91 KOCH WESER, D, DE LA HUERGA, J and POPPER H Effect of choline supplements on fatty metamorphosis and liver cell damage in choline and protein deficiency *J Nutrition* 49, *443 (1953)*
92 RAPPAPORT, A M *Liver Injury* F W Hoffbauer, Editor Trans Eleventh Conf New York Josiah Macy, Jr Foundation, 1952 (p 150).
93 HENCH, P. S Introduction cortisone and ACTH in clinical medicine *Proc Staff Meet, Mayo Clin* 25, *474 (1950)*
94 MARTINI, G A, VON HARNACK, G A and NAPP, J H Hepatitis und Schwangerschaft, die Auswirkung der Hepatitis auf die Mutter *Deutsche med Wchnschr* 78, *661 (1953)*
95 STAUB, H Endémie d hépatite maligne Bale 1946 *Bruxelles-Medical* 48, *2517 (1951)*
96 ——— Haufung schwerster Hepatitisfälle in Basel *Schweiz Ztschr f Path u Bakt* 9, *391 (1946)*
97. ALEXANDER, H D, and ENGEL R W The importance of choline in the prevention of nutritional edema in rats fed low-protein diets *J Nutrition* 47, *361 (1952)*

INDEX

D

E

H

Q

R

Y

Z